



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> :  C12N 15/40, 7/01, 7/04, 15/86, A61K 39/193		A1	(11) International Publication Number: <b>WO 98/53077</b>  (43) International Publication Date: 26 November 1998 (26.11.98)
(21) International Application Number: PCT/US98/10645			(74) Agent: HARRIS, Charles, H.; United States Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, MD 21702 (US).
(22) International Filing Date: 20 May 1998 (20.05.98)			
(30) Priority Data: 60/047,162 20 May 1997 (20.05.97) US 60/053,652 24 July 1997 (24.07.97) US 08/991,840 16 December 1997 (16.12.97) US		US	(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).
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(54) Title: LIVE ATTENUATED VIRUS VACCINES FOR EQUINE ENCEPHALITIS VIRUSES

## (57) Abstract

cDNAs coding for an infectious Western Equine Encephalitis virus (WEE) and infectious Venezuelan Equine Encephalitis virus variant IE (VEE IE) are disclosed in addition to cDNA coding for the structural proteins of Venezuelan Equine Encephalitis virus variant IIIA (VEE IIIA). Novel attenuating mutations of WEE and VEE IE and their uses are described. Also disclosed are attenuated chimeric alphaviruses and their uses.

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TITLE OF THE INVENTION

LIVE ATTENUATED VIRUS VACCINES FOR EQUINE ENCEPHALITIS VIRUSES

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INTRODUCTION

Western equine encephalitis (WEE), eastern equine encephalitis (EEE) and Venezuelan equine encephalitis virus (VEE) are members of the alphavirus genus of the family Togaviridae which is comprised of a large group of mosquito-borne RNA viruses found throughout much of the world. The viruses normally circulate among rodent or avian hosts through the feeding activities of a variety of mosquitoes. Epizootics occur largely as a result of increased mosquito activity after periods of increased rainfall. Western equine encephalitis virus (WEE) was first recognized in 1930 and causes periodic outbreaks of disease in equines. The virus has been detected over much of the western hemisphere from Argentina north to the more temperate regions of central Canada (For a review, see Reisen and Monath [1988] in The Arboviruses: Epidemiology and Ecology, Vol. V. CRC Press, Inc. Boca Raton). Similarly, EEE was first isolated in Virginia and New Jersey in 1933 (Ten Broeck, C. et al. [1935] *J. Exp. Med.* **62**:677) and is now known to be focally endemic throughout much of the northern portion of South

America, Central America and the eastern part of Mexico and the United States. Venezuelan equine encephalitis virus has six serological subtypes (I-VI). Two of these subtypes, I and III have multiple variants, two of these variants are of particular interest in this application, variant IE, and variant IIIA also called Mucambo virus. A live, attenuated vaccine (TC-83) for VEE IA/B has been used for immunization equines and at-risk laboratory and field personnel (Birge et al. [1961] *Am. J. Hyg.* **73**:209-218; Pittman et al. [1996] *Vaccine* **14**:337-343). The vaccine was credited with helping to limit the northward spread of a serious epizootic of VEE originating in South America in the late 1960's.

However, the VEE I/AB vaccines have not yet been licensed by the Food and Drug Administration and have been shown to be effective in preventing disease from VEE IA/B infection only. The current VEE vaccines do not adequately protect against the VEE IE variant or the VEE IIIA variant, as disease has occurred in laboratory workers successfully vaccinated with a vaccine derived from VEE IA/B. In addition, recent unprecedented outbreaks of VEE IE in populations of horses in Mexico indicate a need for a VEE IE vaccine.

The lack of adequate cross protection with existing IA/B vaccines documents the need for a VEE IE-specific and a VEE IIIA-specific vaccine.

The vaccines currently in veterinary use for WEE, EEE and VEE IA/B throughout the United States and Canada are formalin-inactivated preparations. Inactivated vaccines for EEE and WEE are also available for use by at-risk laboratory personnel. These inactivated vaccines are poorly immunogenic, require multiple inoculations with frequent boosters and generally result in immunity of short duration.

The shortcomings of the available vaccines indicate a need for the development of new vaccines of high immunogenicity which induce a longer lasting immunity for protection against WEE, EEE and VEE subtypes IE 5 and IIIA.

#### **SUMMARY OF THE INVENTION**

The present invention satisfies the need mentioned above.

10 In this application are described live attenuated vaccines for WEE, EEE, VEE IE and VEE IIIA which may provide higher level immunity in humans and equines for many years, and possibly for life. In addition, very large numbers of vaccine doses can be produced 15 from significantly less starting materials than is possible with the existing inactivated products. The vaccine preparations of the present invention comprise full-length cDNA copies of the genomes of WEE or VEE IE which have been altered such that the RNA produced 20 from the cDNA, and the virus produced therefrom is attenuated and useful as a live vaccine for human and veterinary use. The vaccine preparations for VEE IIIA and EEE are novel chimeric viruses which include the newly discovered structural protein genes of VEE IIIA.

25 The classic methods of deriving live-attenuated vaccines (blind passage in cell cultures) generally result in heterogeneous and undefined products, hence recent attempts to make live vaccines for alphaviruses have relied on genetic engineering procedures.

30 The alphavirus genome is a single-stranded, positive-stranded RNA approximately 11,400 nucleotides in length. The 5' two-thirds of the genome consist of a non-coding region of approximately 48 nucleotides followed by a single open reading frame of 35 approximately 7,500 nucleotides which encodes the

viral replicase/transcriptase. The 3' one-third of the genome encodes the viral structural proteins in the order C-E3-E2-6K-E1, each of which are derived by proteolytic cleavage of the product of a single open 5 reading frame of approximately 3700 nucleotides. The sequences encoding the structural proteins are transcribed as a 26S mRNA from an internal promoter on the negative sense complement of the viral genome. The nucleocapsid (C) protein possesses autoproteolytic 10 activity which cleaves the C protein from the precursor protein soon after the ribosome transits the junction between the C and E3 protein coding sequence. Subsequently, the envelope glycoproteins E2 and E1 are derived by proteolytic cleavage in association with 15 intracellular membranes and form heterodimers. E2 initially appears in the infected cell as a precursor, pE2, which consists of E3 and E2. After extensive glycosylation and transit through the endoplasmic reticulum and the golgi apparatus, E3 is 20 cleaved from E2 by furin-like protease activity at a cleavage site having a consensus sequence of RX(K/R)R, with X being one of many amino acids present in the different viruses, and with the cleavage occurring after the last arginine residue. Subsequently, the 25 E2/E1 complex is transported to the cell surface where it is incorporated into virus budding from the plasma membrane (Strauss and Strauss [1994] *Microbiological Rev.* **58**: 491-562). All documents cited herein *supra* and *infra* are hereby incorporated in their entirety by 30 reference thereto.

Because the genome of alphavirus is a positive-stranded RNA, and infectious upon transfection of cells in culture, an "infectious clone" approach to vaccine development is particularly suitable for the 35 alphaviruses. In this approach, a full-length cDNA

clone of the viral genome is constructed downstream from a RNA polymerase promoter, such that RNA which is equivalent to the viral genome can be transcribed from the DNA clone *in vitro*. This allows site-directed 5 mutagenesis procedures to be used to insert specific mutations into the DNA clone, which are then reflected in the virus which is recovered by transfection of the RNA.

Previous work with infectious clones of other 10 alphaviruses has demonstrated that disruption of the furin cleavage site results in a virus which incorporates pE2 into the mature virus. Davis *et al.* (1995, *supra*) found that disruption of the furin 15 cleavage site in an infectious clone of VEE is a lethal mutation. Transfection of BHK cells with RNA transcribed from this mutant clone resulted in the release of non-infectious particles. However, a low level of infectious virus was produced which contained secondary suppressor mutations such that virus 20 containing pE2 was fully replication competent and subsequently shown to be avirulent but capable of eliciting immunity to lethal virus challenge in a variety of animal species.

The genetic basis for attenuation of the VEE TC- 25 83 vaccine and certain laboratory strains of VEE virus have been studied extensively and has led to the development of improved live, attenuated vaccine candidates (Kinney *et al.* 1993, *supra*, Davis *et al.* 1995, *supra*). The approach used in this application 30 is similar to that used for VEE, however, following the VEE example exactly did not result in an adequate vaccine for WEE. Changes in the procedure used for VEE were required, none of which could have been predicted from the VEE work, in order to produce the 35 attenuated live WEE virus of the present invention.

Based upon a comparison of the structural protein gene sequences of WEE and other alphaviruses, the probable furin cleavage site of WEE strain CBA/87 virus is RRPKR. The presence of the extra arginine 5 when compared to the consensus (RX(R/K)R) alphavirus cleavage site indicated that the cleavage at this site might be more complex than that observed with VEE virus. It was necessary therefore to prepare two deletion mutations in the E3-E2 cleavage site of the 10 full-length clone, one which lacks five amino acids and one which lacks four amino acids since it was unknown which mutation, if any, would produce an attenuated virus. The residual arginine in the full-length clone lacking only four amino acids was of 15 concern due to the possibility that other mutations might arise due to the presence of the extra arginine resulting in cleavage by cellular proteases at that site and producing an apparently wild type virus with respect to cleavage of pE2.

20 Transfection of cultured cells with RNA transcribed from an infectious clone of WEE lacking the furin cleavage site yielded viruses which contained the pE2 of WEE in the mature virus but which were not replication competent. During intracellular 25 replication of the RNA, mutations arise at low frequency, resulting in a small number of replication competent virus. Sequence analysis of these viruses has shown that the lethal effect of the deletion mutations was alleviated by the appearance of second 30 site mutations in the E2 glycoprotein. These viruses are attenuated in mice when administered by subcutaneous or intracranial inoculation. The mice produce high titer neutralizing and ELISA antibody and are protected against a lethal challenge of parental 35 virulent WEE virus.

Therefore, in one aspect of the invention, the invention pertains to the isolation of a cDNA sequence coding for an infectious western equine encephalitis (WEE) virus RNA transcript. DNA representing the 5 entire genome, not previously available, was prepared by polymerase chain reaction using a series of primer pairs based upon the partial genome sequences previously deposited in Genbank. The 5' and 3' ends of the viral genome were unknown and difficult to 10 obtain. The terminal sequence was necessary for efficient replication of the virus since substitution of ends from a similar virus with similar but not identical sequences resulted in an extremely attenuated virus. In order to determine the correct 15 sequence at the 5' end, a protocol called rapid amplification of cDNA ends (5'-RACE) was used. The full length infectious clone is useful in the production of virulent WEE virus, and introducing and testing attenuating mutations. The production of 20 virulent virus is essential for a formal measure of the degree of attenuation achieved with candidate attenuating mutations and a formal determination of the rate at which reversion to virulence might occur.

In another aspect of the invention, the invention 25 pertains to the isolation of a cDNA sequence coding for an infectious Venezuelan equine encephalitis virus IE variant (VEE IE) virus RNA transcript (SEQ ID NO: 2). Using oligonucleotides specific to genomic RNA of a VEE IE isolate (GenBank accession no. U34999) 30 (Oberste, et al. [1996] *Virology* **219**:314-320), reverse transcriptase polymerase chain reaction was carried out to generate numerous cDNA fragments which were subsequently cloned and used to assemble full-length cDNA of VEE IE. The full length infectious 35 clone is useful, for example, in the production of

virulent VEE IE virus, and introducing and testing attenuating mutations.

In the case of VEE IIIA, the structural protein genes were removed from a full length clone of VEE 5 IA/B and replaced by the VEE IIIA structural genes. The IIIA structural gene sequences were prepared by RT-PCR and include the IIIA 26S promoter and the 3' nontranslated region (3'NTR) flanking the sequences for the structural proteins (SEQ ID NO:3). The VEE 10 IIIA 3' NTR was then replaced with the VEE IA/B NTR, and the modified sequence was cloned back into the VEE IA/B full length clone. The result was a clone in which the nonstructural protein gene sequences were from VEE IA/B and the structural protein gene 15 sequences from VEE IIIA. The virus produced from this chimeric clone replicated efficiently in cell culture, and proved to be completely attenuated in mice. In addition, it was highly immunogenic and protected the vaccinated mice against challenge with virulent, wild- 20 type Mucambo virus (VEE IIIA).

Portions of the cDNA sequences described above are useful as probes to diagnose the presence of virus in samples, and to define naturally occurring variants of the virus. These cDNAs also make available 25 polypeptide sequences of WEE antigens, EEE antigens, VEE IE antigens, and VEE IIIA structural polypeptide antigens encoded within the respective genomes and permits the production of polypeptides which are useful as standards or reagents in diagnostic tests 30 and/or as components of vaccines. Antibodies, both polyclonal and monoclonal, directed against WEE epitopes, EEE epitopes, VEE IE epitopes, or VEE IIIA epitopes contained within these polypeptide sequences are also useful for diagnostic tests, as therapeutic 35 agents, and for screening of antiviral agents.

Accordingly, with respect to polynucleotides, some aspects of the invention are: a purified WEE polynucleotide; a recombinant WEE polynucleotide; a recombinant polynucleotide comprising a sequence 5 derived from a WEE genome or from WEE cDNA; a recombinant polynucleotide encoding an epitope of WEE; a recombinant vector containing any of the above recombinant polynucleotides, and a host cell transfected with any of these vectors.

10 Other aspects of the invention are: a purified VEE IE polynucleotide; a recombinant VEE IE polynucleotide; a recombinant polynucleotide comprising a sequence derived from a VEE IE genome or from VEE IE cDNA; a recombinant polynucleotide 15 encoding an epitope of VEE IE; a recombinant vector containing any of the above recombinant polynucleotides, and a host cell transformed with any of these vectors.

20 In a further aspect of the invention is a complete sequence of the VEE subtype IIIA structural protein genes useful for diagnostics and vaccine development. Also provided is a chimeric virus containing the structural sequences of VEE IIIA, which is completely attenuated and provides protection 25 against challenge with VEE IIIA virulent virus for use as a vaccine

Another aspect of the invention is a single-stranded DNA sequence comprising a cDNA clone coding for an infectious WEE, the cDNA clone including at 30 least one attenuating mutation therein, the RNA produced from transcription of the cDNA and the virus particles produced from the RNA in a host cell for use as a vaccine.

In another aspect of the invention there is 35 provided a full length WEE cDNA clone containing a

defined deletion mutation useful for attenuating the virus for the identification of suppressor mutations in the virus. The attenuated virus with the cleavage deletion and suppressor mutations are useful as a 5 means to generate an attenuated, live WEE virus vaccine for veterinary and human use.

In a further aspect of the invention is provided a chimeric virus containing nonstructural protein gene sequences from WEE and structural protein gene 10 sequences from any alphaviruses including but not limited to Aura, Barmah Forest, Bebaru, Cabassou, Chikungunya, eastern equine encephalitis, Everglades, Fort Morgan, Getah, Highlands J, Kyzylagach, Mayaro, Middelburg, Mucambo, Ndumu, O'nyong-nyong, Pixuna, 15 Ross River, Sagiyama, Semliki Forest, SAAR87, Sindbis, Tonate, Una, Venezuelan equine encephalitis, Whataroa, which could be used as a means for attenuating virulent alphaviruses, and vaccine production against other alphaviruses.

20 Taking advantage of the close evolutionary relationship between WEE and eastern equine encephalitis virus (EEE), a chimeric virus has been constructed in which the structural protein genes of EEE have been inserted into the infectious clone in 25 place of the WEE structural protein genes. The resulting virus is fully replication competent, attenuated, and elicits an immune response in mice which is protective against a lethal challenge with virulent EEE virus.

30 In addition, depending on the non-WEE sequences substituted for the WEE structural genes, another aspect of the invention includes a means for expressing antigens of other alphaviruses as chimeric alphaviruses for use as potential vaccines for human 35 and veterinary use.

In another aspect of the invention there is provided a full length infectious VEE IE cDNA clone containing a cleavage deletion useful in the identification of suppressor mutations in the virus, 5 the RNA produced from the cDNA and the virus produced from the RNA. The virus with the cleavage deletion and suppressor mutations is useful as a means to generate an attenuated, live VEE IE vaccine virus for veterinary and human use.

10 In a further aspect of the invention is provided a chimeric virus containing nonstructural sequences from VEE IE and structural sequences from other alphaviruses which could be used as a means of attenuating virulent alphaviruses.

15 In addition, depending on the non-VEE IE sequences substituted for the structural sequences of VEE IE, another aspect of the invention includes a means to express antigens of other alphaviruses as chimeric alphaviruses as potential vaccines for human 20 and veterinary use.

In a further aspect of the invention, there is provided a vaccine protective against WEE, the vaccine comprising live attenuated WEE virus in an amount effective to elicit protective antibodies in an animal 25 to WEE and a pharmaceutically acceptable diluent, carrier, or excipient.

In yet a further aspect of the invention, there is provided a vaccine protective against VEE IE, the vaccine comprising live attenuated VEE IE virus in an 30 amount effective to elicit protective antibodies in an animal to VEE IE and a pharmaceutically acceptable diluent, carrier, or excipient.

In another aspect of the invention, there is provided a bivalent vaccine protective against WEE and 35 VEE IE, the vaccine comprising both attenuated WEE and

attenuated VEE IE in an amount effective to elicit protective antibodies in an animal to both WEE and VEE IE and a pharmaceutically acceptable diluent. In addition, it is possible that this vaccine will 5 provide short lived protection against other alphaviruses (Cole and McKinney [1971] *Inf. Immunity* 4:37-43).

In yet another aspect of the invention, there is provided an inactivated vaccine produced from the live 10 attenuated virus described above. The attenuated virus of the present invention whether whole virus or chimeric virus can be used in producing inactivated virus vaccines. By using an attenuated virus strain, there is a much greater margin of safety in the event 15 that the produce is incompletely inactivated. Starting with an attenuated strain is also much safer during the manufacturing phase, and allows production under lower biocontainment levels.

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#### BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and appended claims, and accompanying drawings where:

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**Figure 1A, 1B and 1C.** Assembly of the full length cDNA clone of western equine encephalitis virus. Polymerase chain reaction products representing the entire genome of WEE virus were 30 prepared with the primer pairs indicated. Each of the products was cloned into pBluescript KS+. Assembly of the full length clone, pWE2000 in pBluescript was carried out as indicated in the figure and described below. The clones are not drawn to scale. In each of 35 the plasmids, the primers used to generate the PCR

products are indicated as are the restriction endonuclease sites used for the assembly.

5 **Figure 2.** Polypeptide profiles of western equine encephalitis viruses. Samples of purified virus were analyzed by electrophoresis on 10% polyacrylamide gels and stained with Comassie Brilliant blue. Molecular weight marker (MW) are shown in the first lane and molecular weights are indicated in daltons  $\times 10^{-3}$ . The 10 virus strain is designated above the appropriate lane. Position of contaminating bovine serum albumin is indicated by arrow.

15 **Figure 3.** Polypeptide profiles of western equine encephalitis virus (WEE), eastern equine encephalitis (EEE) and chimeric virus, MWE. Samples of purified virus were analyzed by electrophoresis on 10% polyacrylamide gels and stained with Comassie Brilliant blue. Molecular weight markers (MW) are 20 shown and molecular weights are indicated in daltons  $\times 10^{-3}$ . Position of contaminating bovine serum albumin is indicated by arrow.

25 **Figure 4.** Derivation of virulent VEE IE clone. Constructions of cDNA encoding the entire genome of cloned strain 68U201 are shown. The relevant cloning sites and genetic markers are indicated above each clone. The first full-length clone produced that replicated *in vitro* after transfection of the RNA 30 transcribed from the T7 promoter, was pIE-1006. Further modifications to this clone were carried out in order to obtain pIE-1009 that had the *in vitro* and *in vivo* characteristics of the parental, biological isolate, strain 68U201. With a fully virulent clone, 35 pIE-1009 available, specific attenuating mutations

were introduced into pIE-1009 to generate the attenuated clone, pIE1100. Drawings are not to scale. Shaded areas represent regions of pIE1006 that were replaced or mutated to generate new clones.

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**Figure 5.** Construction of IAB-III A cDNA chimeric clone pV3A-1000.

#### DETAILED DESCRIPTION

10 In one embodiment, the present invention relates to a full length cDNA clone of fully virulent WEE virus specified in SEQ ID NO:1, and a full length cDNA clone of fully virulent VEE IE variant specified in SEQ ID NO:2.

15 WEE, strain CBA/87, isolated from the brains of an infected horse in Argentina in 1987 (Bianchi *et al.*, [1987] *Am.J. Trop. Med. Hyg.* **49**:322-328) was used as a parent strain in the instant invention. Any other strain which consistently kills 100% of 5 week old

20 C57BL6 mice when inoculated subcutaneously can be used such as B11, for example. The cDNA clone can be generated by any of a variety of standard methods known in the art. Preferably, DNA representing the entire genome can be prepared by polymerase chain

25 reaction using a series of primer pairs based upon the partial genome sequences previously deposited in GenBank. The 5' terminal sequence of the virus may be determined by 5'-RACE basically as described by Frohman *et al.* ([1988] *Proc. Natl. Acad. Sci. U.S.A.* **85**:8998-9002). Assembly of the full length clone can be in a suitable transcription vector such as, for example, pBluescript KS+, using convenient restriction endonuclease sites or the entire genome could be inserted into any plasmid which contains suitable

30 restriction endonuclease cleavage sites for cloning,

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an origin of replication so that the plasmid can be propagated in a bacterial host, and a selectable marker gene to maintain the plasmid in the bacterial cell during growth. The DNA sequence preferably has a 5 complementary DNA sequence bonded thereto so that the double-stranded sequence will serve as an active template for RNA polymerase. Hence, the transcription vector preferably comprises a plasmid. When the DNA sequence comprises a plasmid, it is preferred that a 10 unique restriction site be provided 3' (with respect to the virion RNA sequence) to ("down-stream" from) the cDNA clone. This provides a means for linearizing the DNA sequence to enhance the efficiency of transcription of genome-length RNA *in vitro*.

15 The complete clone is preferably operatively associated with a promoter region such that the promoter causes the cDNA clone to be transcribed in the presence of an RNA polymerase which binds to the promoter. The promoter is positioned on the 5' end 20 (with respect to the virion RNA sequence), or upstream from, the cDNA clone. An excessive number of nucleotides between the promoter sequence and the cDNA clone will result in the inoperability of the construct. Hence, the number of nucleotides between 25 the promoter sequence and the cDNA clone is preferably not more than eight, more preferably not more than than 5, still more preferably not more than 3, and most preferably not more than 1. Exemplary promoters useful in the present invention include, but are not 30 limited to, T3 promoters, T7 promoters, and SP6 promoters. It is preferable that the 5' end of the *in vitro* transcript not have any additional nucleotides preceding the first nucleotide of the viral sequence. At the 3' end, additional nucleotides can be tolerated 35 in the *in vitro* transcript but are probably lost when

the virus replicates. In most instances, the poly-dA tract at the 3' end is required for viability of the virus. Selection of the virulent full length clone can be achieved by comparing the LD<sub>50</sub> of the virus 5 encoded by the cloned cDNA with the LD<sub>50</sub> of the parent virus used, in the instant example, WEE CBA/87. The ability to produce virulent virus is important; it allows the introduction and testing of attenuation mutations and the attenuated phenotype against a 10 standard: the virulent parent.

Transfection of cells with the RNA transcript coded by the full length genomic cDNA can be achieved by any suitable means, such as, for example, by treating the cells with DEAE dextran, treating the 15 cells with "LIPOFECTIN", and by electroporation. Togavirus-permissive cells, alphavirus-permissive cells, and WEE-permissive and VEE IE-permissive cells are cells which, upon transfection with the viral RNA transcript, are capable of producing viral particles. 20 Togaviruses have a broad host range. Examples of such cells include, but are not limited to, Vero cells, baby hamster kidney cells, chicken embryo fibroblast cells, Chinese hamster ovary cells (CHO), mouse L cells, MRC-5 cells, mosquito cells such as C6-36 25 cells, to name a few.

In the case of VEE IE, an isolate of VEE IE, strain 68U201, was used. Any isolate known to cause disease in man can be chosen. In addition the ability of a strain to have an easily identifiable phenotype 30 such as, for example, the ability to form large plaques in tissue culture on indicator cell monolayers, is helpful. In the present invention, the full length clone of VEE IE was obtained using oligonucleotide primers specific for the VEE IE strain 35 68U201 sequence. Reverse transcription-polymerase

chain reaction (RT-PCR) of strain 68U201 viral RNA was carried out to generate numerous cDNA fragments that were subsequently cloned and used to assemble a full-length cDNA in a plasmid such that the cDNA could be 5 precisely transcribed and viral infectious RNA produced. It is also possible to produce the entire genome by polymerase chain reaction by including the promoter sequence in the 5' end primer thereby producing infectious RNA directly from the PCR 10 fragment. It is also possible to transcribe the viral RNA from a plasmid in the cell by transfection with the appropriate plasmid containing a promoter utilized by cellular RNA polymerases, i.e. the CMV promoter.

To determine virulence of the cloned viral 15 genome, mice can be inoculated subcutaneously with  $10^4$  plaque forming units of the cloned virus. The clone is considered virulent if all mice die, and not fully virulent if mice do not all die. The LD<sub>50</sub> of parent VEE IE strain 68U201 is to 1-2 pfu, therefore, 20 if inoculation of 10,000-fold did not cause lethal disease in all mice, it was considered attenuated.

In another embodiment of the present invention is provided a cDNA sequence of the entire 26S region of VEE subtype IIIA as well as the structural protein 25 genes specified in SEQ ID NO:3.

DNA or polynucleotide sequences to which the invention also relates include sequences of at least about 6 nucleotides, preferably at least about 8 nucleotides, more preferably at least about 10-12 30 nucleotides, most preferably at least about 15-20 nucleotides corresponding, i.e., homologous to or complementary to, a region of the WEE, VEE IE, or VEE IIIA nucleotide sequence. Preferably, the sequence of the region from which the polynucleotide is derived is 35 homologous to or complementary to a sequence which is

unique to the virus. Whether or not a sequence is unique to the virus can be determined by techniques known to those of skill in the art. For example, the sequence can be compared to sequences in databanks,

5 e.g., GenBank. Regions from which typical DNA sequences may be derived include but are not limited to, for example, regions encoding specific epitopes, as well as non-translated regions.

10 The derived polynucleotide is not necessarily physically derived from the nucleotide sequence of the alphaviruses, but may be generated in any manner, including for example, chemical synthesis or DNA replication or reverse transcription or transcription, which are based on the information provided by the 15 sequence of bases in the region(s) from which the polynucleotide is derived. In addition, combinations of regions corresponding to that of the designated sequence may be modified in ways known in the art to be consistent with an intended use. The sequences of 20 the present invention can be used in diagnostic assays such as hybridization assays and polymerase chain reaction assays and for the discovery of other alphavirus sequences.

25 A polypeptide or amino acid sequence derived from the amino acid sequence of alphavirus, refers to a polypeptide having an amino acid sequence identical to that of a polypeptide encoded in the sequence, or a portion thereof wherein the portion consists of at least 2-5 amino acids, and more preferably at least 8-30 10 amino acids, and even more preferably at least 11-15 amino acids, or which is immunologically identifiable with as a polypeptide encoded in the sequence.

35 A recombinant or derived polypeptide is not necessarily translated from a designated nucleic acid

sequence, it may be generated in any manner, including for example, chemical synthesis, or expression of a recombinant expression system.

Once a complete viral genomic cDNA is cloned, 5 attenuation of the virus is possible. An attenuating mutation refers to a nucleotide mutation or amino acid coded for in view of such a mutation which results in a decreased probability of causing disease in its host (i.e., a loss of virulence), in accordance with 10 standard terminology in the art. The attenuating mutation may be a substitution mutation or an in-frame deletion mutation.

Novel WEE attenuating mutations disclosed herein which may be used to carry out the present invention 15 include deletion of five amino acids at the furin cleavage site in combination with a substitution of lysine for glutamic acid at codon 182 at E2, or deletion of five amino acids at the furin cleavage site in combination with a substitution of lysine for 20 glutamic acid at codon 181 at E2. Additionally, certain mutations placed in the non-coding region at the 5' end of the genome have been found to be attenuating, specifically, a C to T change at nucleotide 7, an A to G change at nucleotide 13, a T 25 to A change at nucleotide 25 and deletion of an A at nucleotide 22. These novel attenuating mutations may be inserted together in a cDNA clone encoding WEE virus resulting in an attenuated WEE which is reflected by 100% survival of mice inoculated by 30 subcutaneous and intracranial routes. Such an attenuated live virus is immunogenic and protective against a lethal virus challenge.

Novel attenuating mutations can be discovered in the VEE IE by introducing mutations which are not 35 reparable by the viral RNA replication process. A

preferable mutation is the deletion of the four amino acids of the furin-like cleavage site between the E3 and E2 proteins. Transfection of the mutant viral genome into cells can result in the suppression of the 5 lethal effect of the deletion mutation due to the error prone process of alphavirus replication. Once efficiently replicating viral progeny is generated they can be detected by plaque assays and analyzed for the presence of pE2 protein which indicates that the 10 virus contains the deletion mutation. Attenuated but yet immunogenic virus with a cleavage deletion mutation and suppressor mutation(s) could be tested for its ability to protect animals from challenge with virulent VEE IE.

15 Attenuating mutations may be introduced into cDNAs encoding live WEE or VEE IE by any suitable means, such as site-directed mutagenesis (Please see e.g., Maniatis, Fritsch and Sambrook, Molecular Cloning: A Laboratory Manual (1982) or DNA Cloning, 20 Volumes I and II (D. N. Glover ed. 1985) or Current Protocols in Molecular Biology, Ausubel, F. M et al. (Eds.) John Wiley & Sons, Inc., for general cloning methods.).

25 In another embodiment of the present invention is provided a chimeric virus containing nonstructural sequences from one alphavirus and structural sequences from other alphaviruses which could be used as a means of attenuating virulent alphaviruses. By "Structural sequences" as used herein is meant sequences encoding 30 proteins which are required for encapsidation (e.g., packaging) of the viral genome, and include the capsid protein, E1 glycoprotein, and E2 glycoprotein. By "nonstructural sequences" is meant nonstructural protein sequences, or sequences which encode viral RNA

polymerase(s) proteins. Viruses from which structural sequences can be used in the chimeric virus using WEE "nonstructural genes" as the backbone clone can include for example, all strains of WEE, EEE, and

5 Sindbis, Aura, Barmah Forest, Bebaru, Bijou Bridge, Cabassou, Chikungunya, Everglades, Fort Morgan, Getah, Highlands J, Kyzylagach, Mayaro, Middelburg, Mucambo, Ndumu, O'nyong-nyong, Pixuna, Ross River, Sagiyma, Semliki Forest, SAAR87, Tonate, Una, Venezuelan Equine

10 Encephalitis, Whataroa, to name a few. Acceptable structural protein genes would include a nucleocapsid protein capable of both packaging the chimeric viral genome and which can interact with the glycoproteins to initiate particle assembly. Chimearic virus is

15 constructed by excision of the structural protein genes of the backbone virus and replacement with the desired structural protein genes from another virus. This can be accomplished in one of several ways. For example, site-directed mutagenesis can be used to

20 excise the structural protein genes and leave a restriction endonuclease digestion site at the point of deletion. The structural protein genes of another alphavirus would then be cloned into that restriction site. Any virus obtained after transfection of cells

25 with RNA transcribed from that clone would by definition be a chimeric virus.

In the case where the first and second viruses are closely related, another method can be used wherein cloned structural cDNA sequences of a second

30 alphavirus can be digested at restriction enzyme sites which both viruses have in common. The cDNA fragments of the second virus can then be cloned into the homologous sites in the first virus structural protein locus such that the resulting structural protein genes

35 of the chimeric are a composite of both. Other

methods for producing a chimearic virus are known to people in the art (Kuhn *et al.* [1996] *J. Virology* **70**:7900-7909).

In another embodiment, the attenuated viruses of the present of invention can be used to prepare replicon expression systems. A replicon expression system consists of three components. The first is a replicon which is equivalent to a full length infectious clone from which all of the viral structural proteins have been deleted. A multiple cloning site can be cloned into the site previously occupied by the structural protein genes. Virtually any heterologous gene may be cloned into this cloning site. Transcription of RNA from the replicon yields an RNA capable of initiating infection of the cell identically to that seen with the full-length infectious virus clone. However, in lieu of the viral structural proteins, the heterologous antigen is expressed. This system does not yield any progeny virus particles because there are no viral structural proteins available to package the RNA into particles.

Particles which appear structurally identical to virus particles can be produced by supplying structural proteins for packaging of the replicon RNA in *trans*. This is typically done with two helpers. One helper consists of a full length infectious clone from which the nonstructural protein genes and the glycoprotein genes are deleted. The helper retains only the terminal nucleotide sequences, the promoter for subgenomic mRNA transcription and the sequence for the viral nucleocapsid protein. The second helper is identical to the first except that the nucleocapsid gene is deleted and only the glycoprotein genes are retained. The helper RNA's are transcribed *in vitro* and co-transfected with replicon RNA. Because the

replicon RNA retains the sequences for packaging by the nucleocapsid protein, and because the helpers lack these sequences, only the replicon RNA is packaged by the viral structural proteins and released from the 5 cell. The particles can then be inoculated into animals similar to parent virus. The replicon particles will initiate only a single round of replication because the helpers are absent, they produce no progeny virus particles, and express only 10 the viral nonstructural proteins and the product of the heterologous gene cloned in place of the structural proteins. The heterologous gene product is then detected by the host immune system and appropriate immune response is then mounted.

15 The WEE and VEE IE replicons can be used to express heterologous genes of interest as well as a means for expressing antigens or immunogenic proteins and peptides of interest, *in vitro* or *in vivo*. The immunogenic protein or peptide, or "immunogen" may be 20 any immunogen suitable for inducing an immune response protective against a pathogen from which the immunogen is derived, including but not limited to microbial, bacterial, protozoal, parasitic, and viral pathogens. For example, the immunogen can be the expression 25 product of any heterologous gene of interest, including influenza hemagglutinin, lassa fever nucleocapsid and glycoproteins, portions of bacterial toxin genes, HIV glycoprotein, Ebola glycoprotein, to name a few.

30 In yet another embodiment, the present invention provides inactivated virus vaccines produced from live attenuated virus preparations, either as virus with attenuating mutations as has been described for WEE and VEE IE or chimeric viruses described above for EEE 35 and VEE IIIA. The inactivation of live virus is well

known in the art and can be performed, for example, by the use of formalin. Inactivated attenuated virus vaccine has a greater safety margin both as a final vaccine in case of incomplete inactivation, and during 5 the manufacturing process allowing production under lower biocontainment levels.

Subjects which may be administered the live attenuated or inactivated attenuated viruses and vaccine formulations disclosed herein include both 10 humans and animals (e.g. horse, donkey, pigs, mice, hamster, monkey, birds).

Vaccine formulations of the present invention comprise an immunogenic amount of a live attenuated virus, or a combination of live attenuated viruses as 15 a multivalent vaccine, as disclosed herein in combination with a pharmaceutically acceptable carrier. An "immunogenic amount" is an amount of the attenuated virus sufficient to evoke an immune response, particularly an immune response to the 20 protein or peptide encoded by the heterologous RNA carried by the virus, in the subject to which the virus is administered. An amount of from about  $10^1$  to  $10^5$  plaque forming units of the live virus per dose is suitable, depending upon the age and species of the 25 subject being treated. Exemplary pharmaceutically acceptable carriers include, but are not limited to, sterile pyrogen-free water and sterile pyrogen-free physiological saline solution.

Administration of the live attenuated viruses disclosed herein may be carried out by any suitable means, including both parenteral injection (such as intraperitoneal, subcutaneous, or intramuscular injection), by in ovo injection in birds, and by 30 topical application of the virus (typically carried in 35 the pharmaceutical formulation) to an airway surface.

Topical application of the virus to an airway surface can be carried out by intranasal administration (e.g. by use of dropper, swab, or inhaler which deposits a pharmaceutical formulation intranasally). Topical 5 application of the virus to an airway surface can also be carried out by inhalation administration, such as by creating respirable particles of a pharmaceutical formulation (including both solid particles and liquid particles) containing the virus as an aerosol 10 suspension, and then causing the subject to inhale the respirable particles. Methods and apparatus for administering respirable particles of pharmaceutical formulations are well known, and any conventional technique can be employed.

15 In another embodiment, the present invention relates to antibodies specific for the above-described virus. For instance, an antibody can be raised against any of the viral proteins or against a portion thereof. Persons with ordinary skill in the art using 20 standard methodology can raise monoclonal and polyclonal antibodies to a polypeptide of the present invention. Material and methods for producing antibodies are well known in the art (see for example Goding, in, Monoclonal Antibodies: Principles and 25 Practice, Chapter 4, 1986). The antibodies can be used to monitor the presence or activity of alphaviruses and potentially as a therapeutic agent.

In a further embodiment, the present invention relates to a method of detecting the presence of WEE, 30 EEE, VEE IIIA or VEE IE viral infection or antibodies against these viruses, if present, in a sample. Using standard methodology well known in the art, a diagnostic assay can be constructed by coating on a surface (i.e. a solid support) for example, a

microtitration plate or a membrane (e.g. nitrocellulose membrane), all or a unique portion of WEE, EEE, VEE IIIA or VEE IE virus described above, and contacting it with the serum of a person suspected 5 of having a viral infection. The presence of a resulting complex formed between the virus and antibodies specific therefor in the serum can be detected by any of the known methods common in the art, such as colorimetry or microscopy. This method 10 of detection can be used, for example, for the diagnosis of WEE, EEE, VEE IIIA and VEE IE viral infections.

In yet another embodiment, the present invention relates to a method of detecting the presence of WEE, 15 EEE, VEE IIIA or VEE IE viruses in a sample. Using standard methodology well known in the art, a diagnostic assay can be constructed by coating on a surface (i.e. a solid support) for example, a microtitration plate or a membrane (e.g. 20 nitrocellulose membrane), antibodies specific for WEE and/or VEE IE, and contacting it with serum or tissue sample of a person suspected of having a WEE or VEE IE viral infection. The presence of a resulting complex formed between virus in the serum and antibodies 25 specific therefor can be detected by any of the known methods common in the art, such as fluorescent antibody spectroscopy or colorimetry. This method of detection can be used, for example, for the diagnosis of WEE, EEE, VEE IIIA and VEE IE viral infection.

30 In another embodiment, the present invention relates to a diagnostic kit which contains WEE, EEE, VEE IE, or VEE IIIA virus and ancillary reagents that are well known in the art and that are suitable for use in detecting the presence of antibodies to WEE and 35 VEE IE in serum or a tissue sample. Tissue samples

contemplated can be obtained from birds, monkey, human, or other mammals.

In yet a further embodiment, the present invention relates to DNA or nucleotide sequences for 5 use in detecting the presence or absence of WEE, EEE, VEE IE or VEE IIIA virus using the reverse transcription-polymerase chain reaction (RT-PCR). The DNA sequence of the present invention can be used to design primers which specifically bind to the viral 10 RNA for the purpose of detecting the presence, absence, or quantitating the amount of virus. The primers can be any length ranging from 7-40 nucleotides, preferably 10-15 nucleotides, most preferably 18-25 nucleotides. Reagents and controls 15 necessary for PCR reactions are well known in the art. The amplified products can then be analyzed for the presence or absence of viral sequences, for example by gel fractionation, with or without hybridization, by radiochemistry, and immunochemical techniques.

20 In yet another embodiment, the present invention relates to a diagnostic kit which contains PCR primers specific for WEE, EEE, VEE IE or VEE IIIA and ancillary reagents that are well known in the art and that are suitable for use in detecting the presence or 25 absence of WEE, EEE, VEE IE or VEE IIIA in a sample using PCR. Samples contemplated can be obtained from birds, human, or other mammals.

In another embodiment, the present invention relates to a method of reducing WEE, EEE, VEE IE, or 30 VEE IIIA viral infection symptoms in a patient by administering to said patient an effective amount of anti WEE, anti EEE, anti VEE IE, or anti VEE IIIA antibodies, or protective serum from an immunized animal. When providing a patient with antibodies, the 35 dosage of administered agent will vary depending upon

such factors as the patient's age, weight, height, sex, general medical condition, previous medical history, etc. In general, it is desirable to provide the recipient with a dosage of the above compounds 5 which is in the range of from about 1pg/kg to 10 mg/kg body weight of patient, although a lower or higher dosage may be administered.

In another embodiment, the present invention relates to a method for overcoming vaccine 10 interference in alphavirus-immune individuals. Alphavirus interference has been documented in animals and people since the 1960's. This phenomenon occurs when a live-attenuated vaccine is administered to animals or people with existing immunity to 15 heterologous alphaviruses. Pre-existing immunity may be acquired by vaccination or infection. This presents a significant limitation to the usefulness of the current live-attenuated alphavirus vaccines, especially since the cross-reactive immunity does not 20 protect adequately against challenge with virulent heterologous alphaviruses. Formalin-inactivated vaccines are not an acceptable alternative as they have significant limitations with regard to the quality and duration of protective immunity and 25 require multiple inoculations and periodic boosters. The attenuated WEE, EEE, VEE IIIA and VEE IE virus vaccines of the present invention contain mutations in the viral glycoprotein sequences that may alter the sequence, conformation, and/or accessibility of cross- 30 reactive epitopes. Alterations in epitopes that prevent binding by cross-reactive antibodies may also bypass interference in alphavirus-immune individuals. Eliminating the problem of interference would permit the WEE and VEE IE attenuated virus vaccines to be 35 used in alphavirus-immune animals or people to induce

protective immunity to western equine encephalitis virus and/or to Venezuelan equine encephalitis virus variant IE. Long-lasting protective immunity to both parenteral and aerosol challenge would be expected 5 after vaccination with the live-attenuated vaccines of the present invention, and provide an additional advantage over the use of inactivated vaccines which induce short-lived responses that do not protect against mucosal challenge.

10 Having now described the invention, the following examples are provided to illustrate the present invention, and should not be construed as limiting thereof. In light of the present disclosure, numerous embodiments within the scope of the claims will be apparent to those of ordinary 15 skill in the art.

#### **Attenuated WEE**

20 The following materials and methods were used in the examples that follow.

Viruses and cells. Western equine encephalitis virus, strain CBA/87 (Bianchi et al. 1988) and eastern equine encephalitis virus, strain Fla91-4679 (Mitchell et al. [1992] *Science* 257:526-7) were grown in BHK 25 cells in EMEM containing 10% fetal bovine serum, 100 U/ml penicillin G and 100 µg/ml streptomycin. Primary chicken embryo fibroblasts were grown in EMEM containing 5% fetal bovine serum. Baby hamster kidney cells and Vero cells were grown in EMEM containing 10% 30 fetal bovine serum.

cDNA cloning. Genomic RNA was prepared from purified virus by phenol:chloroform extraction and ethanol precipitation. Initially, cDNA was prepared by the method of Gubler and Hoffman ([1983] *Proc.*

*Natl. Acad. Sci. U.S.A.* **85**:5997-6001). Subsequently, DNA representing the entire genome was prepared by polymerase chain reaction using a series of primer pairs (Table 1, Figure 1) based upon the partial genome sequences previously deposited in GenBank. Each PCR product was cloned into pCRII (Invitrogen). The 5' terminal sequence of CBA/87 virus was determined by 5'-RACE basically as described by Frohman *et al.* ([1988] *Proc. Natl. Acad. Sci. U.S.A.* **85**:8998-9002). The oligonucleotide, CBA/87 5', consisted of an *Sst*II site, the promoter for bacteriophage T7 RNA polymerase followed by one G and 14 nucleotides of the 5' terminus of CBA/87 terminal sequence was paired with ns1962 and used to amplify the terminal 1.9 kb of the WEE genome. Clone pWE2000 representing the entire genome was assembled in pBluescript KS+ through the use of convenient restriction endonuclease sites.

20 Table 1. Oligonucleotide Primers for Preparation of WEE PCR Products

	CBA/87 -T7-Sst2	GTCACCGCGGTAATACGACTCACTATAGATAGGGCATGGTATAGAG (SEQ ID NO:4)
25	NS1962	TCACCTTATTCTGGAACACATCAG (SEQ ID NO:5)
	WEE-7	TCGGAGGAAGGCTGATGAAAC (SEQ ID NO:6)
	WEE-10	TCGGATCCGATGAGAAAATATACGCTCCC (SEQ ID NO:7)
	WEE-17	GACTGGATCCGCAAACCAAGTCCTGTTCTCAGG (SEQ ID NO:8)
30	WEE-18	GCATGGATCCAGCÁTGATCGGAAATGTCTTGTC (SEQ ID NO: 9)
	WEE-5	TCGGATCCACCGCCAAAATGTTCCATAC (SEQ ID NO:10)
	WEE-3	TCGGGATCCCCGGAACATTGGC (SEQ ID NO:11)
	WEE-2	CTGCTTTCATGCTGCATGCC (SEQ ID NO:12)
35	WEE-Not	CGATGCGGCCGCTTTTTTTTTGAAATTAAAAAC (SEQ ID NO:13)
	WEE-CL2	CAGCGTGAAGTCATCGGTAAATGCTGCGTGATGGACATTCAAG (SEQ ID NO:14)
	WEE-CL1	CAGCGTGAAGTCATCGGTAAATGCTTGATGGACATTCAAG (SEQ ID NO:15)

For ease of subsequent site-directed mutagenesis of the structural protein genes, two cassettes representing the 5' terminal 7.6 kb, plasmid pWE5'-18, and 3' 4.2 kb, plasmid pWE3'-17, of the genome were 5 prepared. Full length clones were assembled by digestion of the pWE5'-18 with *BlnI* and *NotI* and insertion of a 4.1 kb *BlnI-NotI* fragment prepared from the plasmid pWE3'-17 or its mutagenized derivatives.

A cassette containing the structural genes of 10 eastern equine encephalitis virus strain Fla91-4679 was prepared by RT-PCR. The cassette was digested with *BlnI* and *NotI* and the 4.0 kb fragment was ligated to pWE5'-18 which had been similarly digested. The resulting plasmid was designated pMWE-7.

15 Mutagenesis of the furin cleavage site. Two oligonucleotides, WEE-CL2 and WEE-CL which bracket the presumed furin cleavage site, RRPKR, between the E3 and E2 glycoproteins were used to delete the 5 and 4 codons, respectively. Plasmid pWE3'-17 was used as 20 template for mutagenesis. WEECL2 and WEECL were paired with primer WEE-5 to generate PCR products of 1.4 kb. The PCR products were purified and paired with WEE-3 for 10 cycles of PCR utilizing WE3'-17 as template. Additional WEE-5 primer was added to 500 nM 25 and PCR was carried out for an additional 20 cycles. The 2.3 kb products were purified, digested with *Bst*II and *Nco*I and ligated into plasmid pWE3'-17 which had been digested with *Bst*II and *Nco*I. Clones containing the mutation were identified by loss of the 30 *Ngo*M1 site in the sequences encoding the furin cleavage site. The sequences of the mutations were confirmed by sequencing and the mutagenized pWE3'-17 cassettes were ligated to pWE5'-18 as described above to yield full length clones pWE2200 and pWE2100, 35 respectively.

Identification of secondary mutations in virus derived from pWE2100 and pWE2200. Virus released from cells electroporated with RNA transcribed from pWE2200 were plaque-purified and grown into stock 5 preparations. Supernatant from cells transfected with RNA transcribed from pWE2100 was diluted 10-fold and inoculated onto BHK cells. The supernatant was collected 48 hours later and the RNA was extracted directly from the culture fluid. For each mutant 10 virus and the parent CBA/87 virus, viral RNA was extracted with Trizol LS (Life Technologies, Inc., Gaithersburg, MD) and the glycoprotein genes of each were amplified by reverse transcription-polymerase chain reaction amplification. The PCR products were 15 purified (PCR Prep, Promega Inc., Madison, WI) and sequenced on an ABI 373 sequencer using fluorescent-tagged terminators.

Transcription and transfection. Purified plasmid was digested with *NotI*, phenol extracted and ethanol 20 precipitated. Typically 0.5-1 ug of linearized DNA was transcribed *in vitro* by T7 RNA polymerase (Ribomax, Promega, Madison, WI) in the presence of 3 mM m7GpppGp (Pharmacia, Piscataway, NJ). Electroporation of BHK or CEF cells with 0.4 ug of RNA 25 was done as described (Liljestrom et al. [1991] *Bio/Technology* 9:1356-1361). The cells were then seeded into T-75 flasks with 20 ml of medium and observed for cytopathology at 24 and 36 hours after electroporation. Virus was harvested when the cells 30 displayed significant cytopathology and approximately 50% were detached from the plastic. Virus titers were determined by plaque assay on Vero and BHK cells.

## EXAMPLE 1

Preparation of an infectious clone of WEE strain CBA/87.

One goal of this study was to prepare a full length cDNA clone of fully virulent WEE virus such that mutations leading to an attenuated phenotype could be identified. WEE, strain CBA/87, isolated from the brain of an infected horse in Argentina in 1987 (Bianchi et al. [1988] *Am. J. Trop. Med. Hyg.* 49:322-328), was chosen as the parent virus as it consistently kills 100% of 5 week old C57BL6 mice when inoculated subcutaneously, allowing development of a convenient animal model to assess the relative effects on virulence of the attenuating mutations. Portions of the sequence of several strains of WEE virus had been determined previously and were used as a basis for primers to prepare amplification products representing the entire genome of the CBA/87 which were cloned in pCRII. Full length clones were assembled in pBluescript KS+ using convenient restriction sites as shown in Figure 1. The first infectious clone pWE1000, contained the 5' terminal 20 nucleotides from eastern equine encephalitis virus. This clone produced viable virus which was highly attenuated exhibiting an subcutaneous LD<sub>50</sub> in mice of approximately 1.2 X 10<sup>6</sup> PFU compared to the CBA/87 parent virus where LD<sub>50</sub> was approximately 22.

A second clone with an authentic WEE 5'-terminus, pWE2000, was used for all subsequent experiments. Transfection of CEF or BHK cells with RNA transcribed from pWE2000 resulted in complete destruction of the monolayers within 36 hours and titers >10<sup>8</sup> PFU/ml were obtained by infection of either cell type with the resulting virus. Subcutaneous inoculation of 5 week

old C57/B16 mice with WE2000 results in death within 9 days and the LD<sub>50</sub> of 75 PFU is only slightly higher than the LD<sub>50</sub> of 22 PFU of CBA/87 parent virus, Table 2. It should be noted that animals surviving the 5 lower doses of virus challenge gave no serological evidence of infection. This level of virulence for WE2000 virus was considered sufficient to allow for further characterization of the mutations necessary for attenuation of the virus.

10

Table 2. C57 Black/6 Mice Inoculated Subcutaneously with CBA/87 Parental virus and WE2000 Recombinant Virus

<u>Virus Strain</u>	<u>Mortality</u>	<u>Mean Day to Death (Days)</u>	<u>Prechallenge ELISA</u>	<u>Challenge (S/T)</u>
<u>CBA/87</u>				
3X10 <sup>5</sup>	10/10	8.4	-	-
3X10 <sup>4</sup>	10/10	8.9	-	-
3X10 <sup>3</sup>	10/10	9.2	-	-
3X10 <sup>2</sup>	7/10	9.9	<100	0/3
3X10 <sup>1</sup>	3/10	10	<100	0/7
3	0/10	--	<100	0/10
<u>WE2000</u>				
1X10 <sup>7</sup>	10/10	8.7	-	-
1X10 <sup>6</sup>	10/10	9.0	-	-
1X10 <sup>5</sup>	10/10	9.2	-	-
1X10 <sup>4</sup>	10/10	9.3	-	-
1X10 <sup>3</sup>	9/10	10.0	<100	0/1
1X10 <sup>2</sup>	3/10	10.0	<100	0/7
1X10 <sup>1</sup>	0/10	-	<100	0/10
1	0/10	-	<100	0/10

15

#### **EXAMPLE 2**

##### Preparation of cleavage mutants of CBA/87.

Davis et al. ([1995] *Virology* 212:102-110) have demonstrated that deletion of the furin cleavage site 20 between E3 and E2 glycoproteins of VEE virus is a lethal mutation. However, prolonged incubation of cells which had been transfected with RNA derived from

full length clones with the deletion, resulted in the eventual appearance of virus which was replication-competent and attenuated in mice (Davis et al., 1995, *supra*). Based upon a comparison of the predicted 5 structural protein sequences of WEE and VEE, the probable cleavage site of CBA/87 virus is RRPKR. The presence of the extra arginine when compared to the consensus (RX(R/K)R) alphavirus cleavage site indicated that the cleavage site of WEE virus might be 10 more complex than that observed with VEE virus. We therefore, prepared two different deletion mutations in the E3-E2 cleavage site of the pWE2000 clone, pWE2100 which lacks five amino acids, RRPKR, and pWE2200 which lacks four amino acids, RPKR.

15 Development of CPE in cells after electroporation of RNA transcribed from pWE2100 and pWE2200 was delayed for 48 to 72 hours compared with pWE2000. In two instances, pWE2100 did not induce significant CPE in the transfected cells despite the fact that 20 approximately  $10^6$  PFU/ml were released into the medium of transfected cells. When assayed by plaque formation on Vero cells, both supernatants yielded extremely small plaques after 72 hours which never increased beyond 2 mm in diameter. In contrast, 25 pWE2000 virus yields large plaques after 48 hours which enlarge to approximately 1 cm after 5 days under a 0.5% agarose overlay. The small plaque phenotype of the mutant viruses is stable after 3 passages in Vero or BHK cells, which is the limit to the passage of the 30 virus used in these experiments.

35 Analysis of the structural proteins of the WE2100 and WE2200 viruses by SDS-PAGE (Figure 2) shows that in each instance, the deletions at the cleavage site result in a virus which lacks E2 protein and contains a larger protein, presumably pE2, indicating that

deletion of the presumed cleavage site eliminated cleavage at this site.

The lack of rapid cytopathology after transfection of BHK cells with transcripts of pWE2200 and pWE2100 suggested that the mutants were non-viable and that the infectious virus subsequently detected by plaque assay was due to secondary mutations arising during the replication of the RNA as reported previously for deletion mutants of VEE (Davis *et al.*, 1995, *supra*).

Three plaque isolates from WE2200 virus were chosen for further characterization. All isolates grew to high titer and exhibited a small plaque phenotype. The isolates were sequenced over the entire glycoprotein reading frame. As shown in Table 3, isolates 2215 and 2220 have a mutation of Glu to Lys at position 181 of the E2 glycoprotein. Strain WE2219 carries a single Glu to Lys at position 182 of the E2 protein. Strain WE2215 also has a conservative Val to Ala change at position 211 of the E2 glycoprotein. WE2220 has a Glu to Gly change at position 2 of the E1 glycoprotein and a Phe to Ser change at position 382 of the E1 glycoprotein.

25 Table 3. Genotypes of Recombinant WEE Virus Strains

	<u>Virus<sup>1</sup></u>	<u>Cleavage Site</u>	<u>E2<sup>2</sup></u>	<u>E1<sup>2</sup></u>
30	CBA/87	RRPKR	P(102), E(181), E(182)	E(2), F(257), P(382)
	vWE2100	-----	K(182)	
	pWE2102	-----	K(182)	
35	vWE2215	R-----	K(181), A(211)	
	vWE2219	R-----	K(182)	
	vWE2220	R-----	K(181)	G(2), S(382)

1. p indicates viruses prepared by mutagenesis of infectious clones.
2. Amino acid at position indicated in parenthesis.

5        In order to determine a consensus of the mutations appearing in virus produced from the pWE2100 RNA, the sequences of the glycoprotein genes were determined directly from cDNA prepared by RT-PCR of the genomic RNA extracted from virus released from BHK 10 cells infected with virus released from the transfected cells. Sequence analysis of glycoprotein genes of WE2100 virus from the transfection supernatant revealed only two mutations. As seen previously in WE2219, WE2100 also had a Glu to Lys 15 change at position 182 of the E2 glycoprotein (Table 3).

      In order to determine which of the mutations identified in virus released from cells transfected with RNA transcripts from pWE2200 and pWE2100 served 20 as the suppressor of the lethal effect of the cleavage deletion mutation, the mutations were individually placed into the pWE2200 and pWE2100 clones by site-directed mutagenesis. As shown in Table 4, three subclones of WE2200 were produced, and based upon the 25 ability to induce CPE in BHK cells, it was demonstrated that the Glu to Lys change at position 181 of the E2 glycoprotein was necessary and sufficient to restore the ability of the WE2200 clone to encode replication competent virus. Similarly, 30 placement of the Glu to Lys change at position 182 of the E2 glycoprotein was also sufficient to restore the ability of the WE2100 clone to encode replication competent virus. When the Glu to Lys changes at E2 position 181 or 182 were inserted into the parental 35 infectious clone pWE2000, the resulting virus

exhibited a small plaque phenotype on Vero cells as noted for each of the cleavage deletion mutants.

5 Table 4. Effect of site-directed mutagenesis on restoration of cytopathogenicity of pWE2200 and pWE2100

	<u>Strain</u>	<u>Cleavage site</u>	<u>E2</u>	<u>E1</u>	<u>Viability</u>
	pWE2000	RRPKR			Yes
10	pWE2200	R----			No
	pWE2221	R----		G(2)	No
15	pWE2222	R----	K(181)	G(2)	Yes
	pWE2223	R----	K(181)		Yes
	pWE2100	-----			No
	pWE2102	-----	K(182)		Yes

20 Although the cleavage deletion mutations in pWE2200 and pWE2100 differed by a single amino acid, the results indicate that the mutations at E2 residues 181 and 182 are both capable of restoring viability of 25 the virus and appear to be equivalent.

### EXAMPLE 3

#### Attenuation of WE2000 virus.

30 C57/BL6 mice are uniformly susceptible to lethal challenge by western equine encephalitis virus until approximately 9 weeks of age. Subcutaneous inoculation of five- or eight-week-old C57/BL6 female mice with CBA/87 or WE2000 viruses routinely results in lethal encephalitis after 8-9 days (Table 2). As 35 noted previously, the WE2000 is slightly less virulent than the CBA/87 parent. The virulence of the progeny virus derived from pWE2100 and pWE2200 infectious clones were determined by subcutaneous inoculation of C57BL6 mice. In each instance, the viruses were

significantly attenuated compared to virus produced from parent WE2000 clone. However, infection of mice with increasing doses of WE2215, WE2219 or WE2220, resulted in sporadic deaths with slightly extended periods prior to death compared to the parental virus (Table 5). These results indicated that deletion of only four amino acids from the cleavage site was inadequately attenuating, unlike the results obtained with VEE virus by Davis *et al.* (1995) and the viruses derived from WE2200 were not characterized further.

Table 5. C57BL6 Mice Inoculated Subcutaneously with WE2200 Cleavage Deletion Mutants

<u>Virus</u> <u>Strain</u>	Mor- tality	Mean day to Death	Prechallenge ELISA	Prechallenge Neut	Chal- leng- e	Post Challenge ELISA	Post Challenge Neut
<b>CBA/87</b>							
$10^3$	10/10	9.1					
$10^5$	10/10	7.7					
<b>WE2000</b>							
$10^3$	10/10	9.2					
$10^5$	10/10	9.1					
<b>WE2215</b>							
$10^3$	1/10	12	504 (3/9)	<20 (9/9)	8/9	8300	761
			100 (6/9)				
$10^5$	1/10	14	253 (7/9)	<20 (9/9)	9/9	12800	403
			100 (2/2)				
<b>WE2219</b>							
$10^3$	0/10	-	283 (2/8)	<20 (10/10)	6/10	14368	2281
			100 (6/8)				
$10^5$	2/10	12	200 (4/8)	<20 (8/8)	8/8	11738	1522
			100 (4/8)				
<b>WE2220</b>							
$10^3$	1/10	11	606 (5/9)	20 (3/9)	5/9	14703	1280
			100 (4/9)	<20 (6/9)			
$10^5$	2/10	14	800 (7/8)	20 (2/8)	8/8	9051	761
			100 (1/8)	<20 (6/8)			

When C57BL6 mice were inoculated subcutaneously with the uncloned WE2100 progeny virus, there were no deaths at any dilution, even at doses of  $10^7$  PFU per mouse (Table 6). However, some of those mice inoculated subcutaneously with  $10^5$  PFU or less remained susceptible to a lethal challenge with the virulent CBA/87 virus.

10 Table 6. C57 Black/6 Mice Inoculated Subcutaneously with WEE Virus Strain WE2100

Virus strain	Mortality <sup>1</sup>	Mean Day to Death	Challenge <sup>2</sup>
Dose	(%)		(%)
<u>WE2100</u>			
$10^3$	0/10 (0)	-	4/10 (40)
$10^4$	0/10 (0)	-	7/10 (70)
$10^5$	0/10 (0)	-	8/10 (80)
$10^6$	0/10 (0)	-	10/10 (100)
$10^7$	0/10 (0)	-	10/10 (100)

15 1. Expressed as animals dying/animals tested  
 2. Expressed as animals surviving/animals tested

WE2102 virus was demonstrated to be highly attenuated and killed only two of twenty mice when inoculated subcutaneously with the highest dosage of virus ( $10^7$  PFU). All mice were challenged 3 weeks later with  $10^5$  PFU of CBA/87 virus. Mice previously immunized with  $10^5$  PFU or more of WE2102 survived with no noticeable symptoms. Thus, an effective immunizing dose of WE2102 is at least 100 fold less than that required to kill C57BL6 mice. These results further indicate that the Glu to Lys change at position 182 of the E2 glycoprotein is responsible for restoring viability to viruses containing a deletion of the furin cleavage site in the WEE glycoproteins and that WE2102 virus is an effective attenuated vaccine virus.

Table 7. Subcutaneous Inoculation of C57BL/6 Mice with recombinant WEE virus strain 2102 confers protection against a lethal challenge

5

<u>virus</u> <u>Strain</u>	<u>Mortality<sup>1</sup></u> (%)	<u>Mean Day</u> <u>to Death</u>	<u>Challenge<sup>2</sup></u> (%)
Dose			
<b>WE2100</b>			
10 <sup>3</sup>	0/10 (0)		3/10 (30)
10 <sup>5</sup>	1/10 (10)	16	9/9 (100)
10 <sup>7</sup>	0/10 (0)		10/10 (100)
<b>WE2102</b>			
10 <sup>3</sup>	0/20 (0)	-	9/20 (45)
10 <sup>5</sup>	0/20 (0)	-	18/20 (90)
10 <sup>7</sup>	2/20 (0)	14	18/18 (100)
<b>WE2000</b>			
10 <sup>5</sup>	10/10 (100)	9.2	

1. Expressed as animals dying/animals tested  
 2. Expressed as animals surviving/animals tested

10

#### Attenuated VEE IE

##### EXAMPLE 4

Sequence Analysis. Genomic RNA from VEE IE Strain 68U201 was isolated and reverse transcription of the genomic RNA followed by polymerase chain reaction (RT-PCR) was used to generate cDNA of the virus. Initial sequencing of the strain 68U201 genome employed oligonucleotide primers based on existing VEE IE sequence and VEE IA/B sequence. After a partial sequence of strain 68U201 was determined, oligonucleotides specific to the strain 68U201 sequence were used to obtain a complete sequence of the strain 68U201 viral genome. The exact 5' end of the genome was determined by PCR/RACE technique (Frohman et al. [1988] Proc. Natl. Acad. Sci. U.S.A. 85:8998-9002). The entire strain 68U201 viral genome consists of 11,464

15

20

nucleotides, excluding the poly-A sequence (Oberste *et al.* [1996] *Virology* **219**: 314-320).

EXAMPLE 5

5 Construction of Full-length, Live Clones

Using oligonucleotides specific to the VEE IE strain 68U201 sequence, RT-PCR of strain 68U201 viral RNA was carried out to generate numerous cDNA fragments that were subsequently cloned. These clones 10 were used to assemble a full-length cDNA of strain 68U201 in a plasmid situated such that the cDNA could be precisely transcribed in an *in vitro* transcription reaction employing T7 polymerase. The first 15 nucleotide downstream of the T7 promoter is a G followed by a cDNA encoding the entire strain 68U201 genome, including poly-A sequence. For the purposes of run off *in vitro* transcription, a unique 20 endonuclease restriction site (*NotI*) follows the poly-A sequence (Figure 4). DNA sequences encoding the T7 promoter and the strain 68U201 genome were cloned into a suitable plasmid for propagation and selection in *E. coli*. Oligonucleotides relevant to construction of 25 full-length infectious clones are shown in Table 8.

25 Table 8

0077	CTAAGAGGGGCCCTATATC (SEQ ID NO:16)
0111	GCGGAATTCTAATACGACTCACTATAGATGGCGGCGCATGAGAG (SEQ ID NO:17)
30 0113	TGACCGCGGGACCTCTGTCCAC (SEQ ID NO:18)
0126	AAGTGCATCGATTCAGCG (SEQ ID NO:19)
0136	CTGAAATGTCCAGGATCCACGGAGGAGCTG (SEQ ID NO:20)
0137	CAGCTCCTCCGTGGATCTGGACATTCAG (SEQ ID NO:21)
35 0140	GACTGCGGCCGCTTTTTTTGAAATATTAAAAACAAAATCC (SEQ ID NO:22)
0220	CGAGAATCGATGCACTTCAGCC (SEQ ID NO:23)

Descriptions of oligonucleotides are as follows: 0077 introduces an *Apa*I endonuclease restriction site along with a serine to proline mutation within the coding region of nsP4. 0111 encodes an *Eco*RI 5 endonuclease restriction site followed by the T7 promoter, a single G, and the first 18 nucleotides of the VEE IE genome. 0126 introduces a *Clai*I endonuclease restriction site within the coding region of nsP3 and was used in combination with 0220. 0113 10 was used to remove an *Apa*I endonuclease restriction site within the structural genes. 0140 encodes a unique *Not*I endonuclease restriction site followed by a 12 T's, and the reverse complement of the last 21 nucleotides of the VEE IE. A *Not*I site at the end of 15 the cDNA encoding the VEE IE genome allowed for run-off transcription after digestion of the plasmid with *Not*I. 0220 introduces a *Clai*I endonuclease restriction site within the coding region of nsP3 and was used in combination with 0126. Minor alterations of the 20 nucleotide sequence using 0077, 0113, 0126, and 0220 facilitated assembly of the full-length clones and allowed for rapid diagnostic analysis of virus generated from these clones by RT/PCR methods.

The full-length clone obtained, pIE1006, was 25 transcribed *in vitro* using T7 polymerase of *Not*I-linearized plasmid and the RNA transfected into BHK-21 cells (Figure 4). The phenotype of the resulting virus was markedly different from the parent virus (strain 68U201) from which the cDNA was derived. The 30 virus derived from pIE1006 *in vitro* transcribed RNA, VIE1006, gave rise to small plaques upon infection of target monolayers (Table 9). In attempts to recover the phenotypic characteristics of strain 68U201, regions of the pIE1006 clone were replaced with cDNA 35 generated by RT-PCR from strain 68U201 RNA. Three

subsequent full-length, live clones were constructed by replacing pIE1006 sequences with 928, 4492, and 8130 nucleotides generated by RT-PCR of strain 68U201 RNA. These clones were designated pIE1007, pIE1008 and pIE1009; and viruses derived from these clones were designated VIE1007, VIE1008 and VIE1009, respectively. These clones are shown in Figure 4. The relevant cloning sites and genetic markers are indicated above each clone. Numbers indicate the corresponding nucleotides in the strain 68U201 genome. The T7 promoter is shown. Drawings are not to scale. Shaded areas represent regions of pIE1006 that were replaced or mutated to generate new clones. The characteristics of these viruses are described below.

15

#### EXAMPLE 6

Assessment of Viruses Derived from Various Clones. The viruses VIE1006, VIE1007, VIE1008 and VIE1009 were derived from plasmids pIE1006, pIE1007, pIE1008, and pIE1009, respectively. Analysis of virus derived from these clones by plaque assay is shown in Table 9. Plaque size was determined by infection of Vero cell monolayers followed by agarose overlay.

Analysis of virus derived from these clones (Table 9) by plaque assay indicated that VIE1007 gave the same plaque morphology as VIE1006 and therefore was not studied further. The VIE1008 produced larger plaques in comparison to VIE1006, and the VIE1009 virus gave rise to the largest sized plaques of the four viruses tested. Plaques generated by VIE1009 were similar to those produced by the parental virus, strain 68U201.

Table 9: In vitro analysis of virus derived from molecular clones of VEE IE strain 68U201

	<b>Virus Strain<sup>1</sup></b>	<b>Plaque Size</b>
5	<b>VIE1006</b>	small
	<b>VIE1007</b>	small
10	<b>VIE1008</b>	medium
	<b>VIE1009</b>	large
	<b>VEE IE 68U201</b>	large

**EXAMPLE 7**

15        In Vivo studies of VEE IE Vaccine Candidates. The distinct plaque morphologies of VIE1006, VIE1008, and VIE1009 suggested that these viruses may behave differently *in vivo*. To assess the relative virulence of the cloned derivatives of strain 68U201, mice were 20 initially inoculated with subcutaneously VIE1006, VIE1008, and VIE1009 viruses (Table 10). Mice infected with VIE1006 and VIE1008 were not adversely affected by these viruses as assayed by the number of mice surviving the infection. VIE1009 proved to be as 25 virulent as strain 68U201, causing death in all of the animals infected (Table 10). Immunogenicity of the different viruses inferred by the demonstration of a protective immune response and was determined by back challenge of surviving animals in the virulence assay 30 with approximately  $10^4$  pfu of the virulent, parental virus, strain 68U201. Back challenge was performed four weeks after the initial inoculation.

Table 10: In vivo analysis of virus derived from molecular clones of VEE IE strain 68U201

5	<b>virus</b>	<b>Strain<sup>1</sup></b>	<b>Mortality<sup>2</sup></b>	<b>Challenge<sup>3</sup></b>
	<b>VIE1006</b>	0/10	10/10	
	<b>VIE1008</b>	0/10	10/10	
	<b>VIE1009</b>	10/10	nd	
10	<b>VEE IE 68U201</b>	nd		1/10

<sup>1</sup> Initial inoculation with approximately  $10^4$  PFU of each virus with the exception of VEE IE 68U201 which were left untreated until challenge phase of experiment.

<sup>2</sup> Expressed as animals dying/animals tested.

<sup>3</sup> Expressed as animals surviving/animals tested. All animals were challenged with approximately  $10^4$  PFU of VEE IE strain 68U201 four weeks after the initial inoculation.

#### **EXAMPLE 8**

Construction of a Full-length, Molecularly Attenuated VEE IE Clone. With the availability of a full-length virulent clone, specific attenuating mutations were introduced into the structural genes of the virus by site-directed mutagenesis. A deletion mutation was used instead of a point mutation because of the inability of viral RNA replication to repair such mutations. The glycoproteins of VEE IE are produced as a poly-protein precursor, PE2. The junction between the E3 and E2 proteins is cleaved by a furin-like cellular protease. The amino acid sequence of the presumed furin-like protease cleavage site of strain 68U201 is RGKR. The nucleotides encoding these four amino acids of the furin-like cleavage site between the E3 and E2

proteins were deleted from the pIE1009 clone and the resulting "cleavage deletion" clone was designated pIE1100. The oligonucleotides used to generate the cleavage deletion mutation are shown in Table 8.

5 Oligonucleotide 0136 encodes a cleavage deletion mutation that eliminates the four amino acid furin-like cleavage site found in PE2 and was used in combination with oligonucleotide 0137. 0137 encodes the reverse complement of 0136.

10 Transfection of RNA transcribed from pIE1100 into tissue culture cells required extended incubation periods before viral cytopathic effect became apparent in the cultured cells. This extended incubation period is indicative of transcripts from full-length  
15 clones possessing mutations that partially inhibit viral replication. However, such mutations can be suppressed by second-site mutations which arise randomly via the error prone process of alphavirus replication, resulting in variants with enhanced  
20 replication ability. In fact, the culture media from cells transfected with RNA transcribed from pIE1100 contained low titers of infectious virus, VIE1100, which could be amplified to high titer ( $4 \times 10^7$  PFU/ml) upon subsequent passage. Biochemical analysis showed  
25 that this virus had an uncleaved PE2 protein indicating that the cleavage deletion mutation totally prevented proteolytic processing of the surface E2 glycoprotein precursor. The efficacy of VIE1100 virus to serve as a vaccine was evaluated in mice as  
30 described below.

One specific suppressor mutation is thought to reside at nucleotide 10,181 of the VEE IE genome, a C to U nucleotide substitution resulting in an amino acid change from Serine to Leucine in the E1 protein position #57. Ability of this mutation to suppress the

lethal nature of the furin cleavage site deletion mutation was assessed. The cDNA of pIE1100 was mutated by changing nucleotide 10,181 from a C to T. This introduced a serine to leucine change at amino acid number 57 of E1, a mutation found within the stock of VIE1100. The resulting clone was designated pVIE1150. Transfection of RNA from pVIE1150 into BHK-21 cells lead to the production of approximately  $10^6$  PFU/ml of virus in the supernatant of the transfected cells at 48 hours post-transfection.

**EXAMPLE 9**

Vaccine Studies with a Full-length, Molecularly Attenuated Virus. Balb/C mice were inoculated with VIE1100 virus at various doses ( $10^4$ ,  $10^6$ , and  $10^7$  PFU per mouse), and was found to be completely attenuated at a dose 100,000 times higher than that required to cause lethal disease by the parent virus, strain 68U201 (Table 10). Subsequent challenge of these animals with virulent strain 68U201 demonstrated that immunization with VIE1100 virus provided complete protection from lethal virus challenge (Table 11).

Table 11: Vaccination study with VIE1100

	<b>Virus Strain</b>	<b>Mortality<sup>1</sup></b>	<b>Challenge<sup>2</sup></b>
	<b>Dose</b>		
<b>VIE1100</b>			
30	$10^4$	10/10	10/10
	$10^6$	10/10	10/10
	$10^7$	10/10	10/10
	Mock Vaccinated	0/10	0/10

35 1. Expressed as animals dying/animals tested.

2. Expressed as animals surviving/animals tested after inoculation with  $10^4$  PFU of VEE IE strain 68U201

**EXAMPLE 10**

Properties of a WEE-EEE chimeric virus. Based upon similarities of amino acid sequences of the carboxy terminal portion of NSP-4 and the amino 5 terminal portion of the capsid proteins of WEE, EEE and Sindbis virus, it has been suggested that WEE virus arose by recombination between EEE and a Sindbis virus ancestor (Hahn et al. [1988] *Proc. Natl. Acad. Sci. U.S.A.* **85**: 5997-6001). The capsid genes of both 10 WEE and EEE contain a highly conserved sequence with a unique Bln I site 76 nucleotides downstream of the initiation codon. We utilized this site to insert the structural protein sequences of EEE into pWE5-18 in order to construct a full length clone encoding a 15 chimeric virus. Plasmid pMWE-7 is a full length clone consisting of the 5' non-coding sequence, nonstructural genes, 26S promoter and the first 25 codons for the capsid protein of WEE CBA/87 fused to the structural protein genes and 3' non-coding 20 sequence of EEE strain Fla91-4679. Transfection of BHK cells with RNA transcribed from pMWE-7 resulted in complete destruction of the monolayer and release of high yields of virus. SDS-polyacrylamide gel 25 electrophoresis of the purified virus demonstrated that the virus is composed of polypeptides which comigrate with those of EEE and not of WEE, indicating that the virus is a chimera (Figure 3).

Injection of mice with the MWE chimeric virus killed mice only sporadically, suggesting that fusion 30 of the sequences of the two viruses resulted in significant attenuation compared to the parent WE2000 virus (Table 12). Mice immunized with MWE-7 developed significant neutralizing antibody titers and resisted a lethal EEE Fla91-1467 challenge when 35 immunized with greater than  $10^5$  plaque forming units of

MWE-7 virus. The neutralizing antibody response after challenge was not significantly elevated indicating that the immunization with the chimeric virus effectively prevented infection by a lethal EEE 5 challenge. Therefore, a chimeric virus derived by combining the structural protein genes of EEE with the non-structural proteins genes of WEE may serve as a safe, effective approach to development of a vaccine for EEE virus.

10

Table 12. Immunization of C57BL6 Mice with an WEE/EEE Chimeric Virus Confers Protection Against a Lethal EEE Challenge

<u>virus</u> <u>strain</u>	<u>Mortality<sup>1</sup></u>	<u>Mean Day to Challenge<sup>2</sup></u>	
Dose		Death	
<u>WE2000</u>			
10 <sup>5</sup>	10/10 (100)	8.5	
<u>MWE7</u>			
10 <sup>3</sup>	0/10 (0)		6/10 (60)
10 <sup>5</sup>	1/10 (10)	8.0	9/9 (100)
10 <sup>7</sup>	0/10 (0) <sup>3</sup>		9/9 (100)
<u>Saline</u>	0/10 (0)		0/10 (0)
<u>Control</u>			

15

1. Expressed as animals dying/animals tested
2. Expressed as animals surviving/animals tested
3. One animal died during pre-challenge bleed

20

Example 12  
VEE IIIA Chimera

Construction of IAB-IIIa cDNA Chimeric Clone (pV3A-1000)

25 BeAn8 (wild type Mucambo or VEE IIIA) cDNA was amplified with primers 128 and 140 (Table 13) to generate a PCR fragment of the entire 26S region of BeAn8. Primer 128 incorporated an ApaI site just upstream of the BeAn8 26S promoter to facilitate 30 cloning into pV3000. Primer 140 introduced a NotI

site just downstream of the poly(A) tract which would also facilitate cloning into pV3000 as well as enable run-off transcription. This 128/140 PCR fragment was cloned into pBluescript SK+ vector using the ApaI and 5 NotI sites and was termed pMUC-1000.

To replace the BeAn8 3' nontranslated region (3'NTR) in pMUC-1000 with the corresponding region in pV3000, an EcoRI site was introduced immediately downstream of the E1 stop codon in both pMUC-1000 and 10 pV3000 using the primer pairs 013/227 and 225/226, respectively. The 013 primer is a universal primer found in the pBluescript SK+ vector and the 227 primer was designed to introduce an EcoRI site in pMUC-1000. The 225 primer introduced an EcoRI site in pV3000 15 and the 226 primer is located in the vector, TotoX. The 013/227 and 225/226 PCR fragments were separately cloned into the pBluescript SK+ vector producing pMUC-1200 and pV3nt-1000, respectively. The 3' NTR encoded in pV3nt-1000 was shuttled into pMUC-1200 using the 20 EcoRI site and the NotI site to produce pMUC-1300.

The full-length chimeric cDNA clone, pV3A-1000, was constructed by shuttling the structural genes encoded in pMUC-1300 into the pV3000 nonstructural 25 domain using the ApaI site and NotI site.

Table 13. Primers for construction of the pV3A-1000

013 -- AACAGCTATGACCATG (SEQ ID NO:24)  
128 -- CTGAGAGGGGCCAGTAAC (SEQ ID NO:25)  
30 140 -- GACTGCGGCCGCTTTTTTTTGAAATATTAAAAAA (SEQ ID NO: 26)  
225 -- CCAGAACATAATTGAATTCAAGCAATTG (SEQ ID NO:27)  
226 -- CTTTATCCGCCTCCATCC (SEQ ID NO:28)  
227 -- CCAATCGCTGCTGAATTCTAATTATGTTCTG (SEQ ID NO:29)  
35

Five week old C57BL6 mice were used in a challenge study (Table 14). All mice immunized with

10<sup>7</sup> PFU of pV3A-1000 produced neutralizing antibodies. Mice were challenged with 10<sup>7</sup> PFU of 900807 (a Mucambo virus from Trinidad). The geometric mean neutralizing antibody titer in mice inoculated with 10<sup>7</sup>, 10<sup>5</sup>, and 5 10<sup>3</sup> PFU of pV3A-1000 was 9800, 8800, and 7000 respectively.

10 Table 14. Results from challenge studies

	Fraction of mice sick/inoc	Fraction of mice dead/inoc	Average days to onset of illness	Average days to death
15 Immunized with pV3A-1000	0/20	0/20	N/A	N/A
20 Unvac- cinated	19/20	12/20	6.2	10.5

25

30

35

40

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5 (i) APPLICANT: Michael D. Parker  
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10

(ii) TITLE OF INVENTION: Live Attenuated virus  
vaccines for western equine encephalitis virus,  
eastern equine encephalitis virus, and venezuelan  
equine encephalitis virus IE and IIIA variants

15

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30 (A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: Apple Macintosh  
(C) OPERATING SYSTEM: Macintosh 7.5  
(D) SOFTWARE: Microsoft Word 6.0

35

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

40 (vii) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER: Provisional  
Application 60/047162,  
(B) FILING DATE: May 20, 1997

(A) APPLICATION NUMBER: Provisional  
Application 60/053,652

(B) FILING DATE: July 24, 1997

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(2) INFORMATION FOR SEQ ID NO:1:

15 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11492 base pairs  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear

20 (ii) SEQUENCE DESCRIPTION: SEQ ID NO:1:

1 ATAGGGCATG GTATAGAGGC ACCTACCCTA CAAACAAATC  
GATCCAATAT

25 51 GGAAAGAATT CACGTTGACT TAGACGCTGA CAGCCATAT  
GTCAAGTCGT

101 TACAGCGGAC GTTTCCACAA TTTGAGATCG AAGCAAGGCA  
GGTCACTGAC

30 151 AATGACCATG CCAATGCCAG AGCGTTTCG CATGTGGCAA  
CAAAGCTCAT

35 201 TGAGAGCGAA GTCGACCGGG ACCAAGTTAT CTTGGACATT  
GGAAGTGC

251 CCGTCAGACA TGCACATTCC AACCAACCGCT ATCATTGTAT  
CTGCCCATG

40 301 ATAAGCGCTG AAGACCCGGA CAGACTACAG CGGTATGCAG  
AAAGACTTAA

351 GAGAAGTGAC ATGTACCGAC AAGAATATAG CCTCTNAAGG  
CGGCAGACCT

45 401 GCTGGAAGTC ATGTCCACAC CAGACGCAGA GACTCCATCT  
CTGTGTATGC

451 ACACAGACGC CACGTGTAGG TACTTTGGAA GTGTANGCAG  
TATAACCAAGA

501 TGTGTACGCA GTCCATGCAC CGACATCAAT CTACCACCAAG  
GCGCTTAAAG \*

5 551 GAGTTAGGAC AATTACTGG ATAGGTTTG ACACGACCCC  
TTTTATGTAC

10 601 AAAAACATGG CAGGTTCCCTA CCCTACTTAC AACACAAACT  
GGGCCGACGA

15 651 GAGAGTATTG GAAGCACGTA ACATTGGCCT CGGTAACTCA  
GATCTTCAGG

20 701 AGAGCAGACT TGGAAAAC TT TCAATCCTTA GGAAGAAGAG  
GCTCCAACCT

25 751 ACTGATAAGA TCATATTCTC GGTTGGTTCA ACAATCTACA  
CAGAGGATAG

30 801 ATCACTGTTA CGTAGCTGGC ATCTTCCAAA CGTGTCCAC  
CTGAAAGGAA

35 851 AGTCTNACTT CACAGGTAGA TGTGGGACCA TTGTCAGCTG  
TGAAGGGTAT

40 901 GTCATCAAAA AGATAACGAT CAGCCCAGGA CTATACGGTA  
AAGTTGAGAA

45 951 CTTGGCGTCC ACGATGCATC GCGAGGGTTT CTTGAGTTGC  
AAAGTCACAG

50 1001 ATACGCTGCG CGGCGAGAGG GTTTCTTTG CTGTGTGTAC  
GTATGTACCA

55 1051 GCCACACTTT GCGATCAGAT GACAGGGAGG GCTCAACCAA  
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60 1101 TCAATGGTAG GACGCCAAGA AATACTNACA CAATGCAGAA  
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15 1551 CCCTGAAATA GAAAAAGAGA CCGTAGAGGC AGAAGTAGAC  
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30 11151 TGCTTATAAA CACACGTAGA TGACTGAGCG CGGACACTGA  
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30 11201 AAAACTCGAT GTACTTCCGA GGAAGCGTGG TGCATAATGC  
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35 11251 TTGACACTAA AACTCGATGT ATTTCCGAGG AAGCACAGTG  
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45 11401 ATTTAAAAAC ACTACACACA CTTTATAAAT TCTTTATAA  
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## (3) INFORMATION FOR SEQ ID NO:2:

5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 11464 base pairs  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Unknown

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15 181 GGCATCGAAA TTGATCGAGA CGGAGGTGGA ACCATCCGAT  
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25 481 AGTCGCAGTG TATCAGGATG TGTACGCGGT TGACGGACCG  
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30 GCCGACGAGA CCGTGTAAAC  
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2221 GAAGTCTGGA ATTATTAAGA GCGCAGTCAC AAAGAAGGAC  
TTGGTAGTGA GTGCTAAGAA

15 2281 GGAGAACTGC GCCGAGATAA TAAGGGACGT CAAGAAGATG  
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2341 CCGGACGGTG GACTCAGTGC TGTTAAATGG ATGCAAGCAC  
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2401 TGATGAAGCC TTTGCATGCC ACGCCGGCAC TCTCAGGGCC

20 TTAATAGCCA TTATACGCC

2461 AAAGAAAGCA GTGCTATGTG GTGACCCAAA GCAATGTGGC  
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25 2521 GAAAGTGCAC TTCAACCATG AAATATGCAC TCAAGTTTC  
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2581 GTGTACCAAG TCAGTGACGT CGGTAGTGTGTC CACACTGTTT  
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2701 GGATGACTTG ATTCTCACAT GTTTAGGGG ATGGGTTAAG

30 CAACTGCAA TAGACTACAA

2761 AGGAAATGAA ATAATGACCG CGGCTGCCTC ACAGGGATTG  
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2821 AGTTAGGTAC AAAGTTAACG AGAACCCATT GTACGCACCC  
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2881 GCTGTTGACC CGGACGGAAG ACAAGATTGT GTGGAAGACT  
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5 3001 AGAACATGAT GCCATCATGA GACACATCCT GGAGAAACCG  
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3121 AGATTTGACC ACAGAGCAGT GGAACACAGT GGATTACTTC

10 10 AAAGAGGATA AGGCCCACTC

3181 AGCTGAGATT GTCCTGAATC AGCTGTGCGT GCGATTCTTC  
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15 15 3301 GTCACCCAAAC ATGTACGGGT TGAATCAAGA AGTGGTCAGG  
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3421 CAATTATGAT CCGCGCATAA ATTTAGTACC GGTGAACCGT

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25 25 3601 ATCTGAAACA CCTGATTCTA CTTTTAGGGC GCGCCTAGAT  
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30 30 AAGAAGGCCT GCCTGCACCT

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25 4621 TAGCGATGGG AAGACATTCT CATATCTGA AGGAACCAAA  
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30 TGCCCCGTTG AGGAGTCAGA  
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10 10 AGCGCAAGTG CTCAATCTTC

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5 9121 GGTAGTTCTG TTCATGTGAC TCCACCTGCC GGGCAAAGCG  
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15 9421 GTGTCTCTGA AACATTCATCC TAAGAACCCC ACCTTCCTAA  
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5 10141 CTTGAGTACA TTACATGCCA TTACAAGACT GGAATGGATT  
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10 ATACGGAGAA TACCCAGATA

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15 10441 GCAGTGGTGT ATGTGAATGG AGAGACTCCC GTTAATTAA  
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10561 ATTTATAACT ATGACTTTCC GGAATATGGA GCCGGCCACG

20 CAGGAGCGTT TGGTGACATC

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25 10741 AAGAAAGATA AACCAACCATC CCTCAAGTTC ACAGCCCCGT  
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10921 AACGAGTGTG TATATTACATC CGACTTTGGC GGGATCGCTA  
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10981 AAGTCAGGCA AATGTGCAGT TCATGTACCC TCAGGCACGG  
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11041 GTCGAGTTGG CCGAACAGGG TTCGGCTACT ATACATTTT  
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 5 11161 AAAGATCACA TTGTGACGCA TCCCCAATAC CACGCCAGT  
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 11281 GGACTTGTGT TAGCCACAGT TGTGGCTATG TATGTGCTGA  
 10 CCAACCAGAA ACATAATTAG  
 11341 TATTAGCAGC GATTGGCATG CTGCTTGTAA AGTTTTATTA  
 CAAATAACGT GCGGCAATTG  
 11401 GCGAGCCGCT TTAATTAGAA TTTTATTTTC TTTTACCATTA  
 ATTGGATTTT GTTTTTAATA  
 15 11461 TTTC

## (4) INFORMATION FOR SEQ ID NO:3:

20 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 4003 base pairs  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Double  
 (D) TOPOLOGY: Unknown  
 25 Nucleotide Sequence of the BeAn8 26S Subgenomic  
 cDNA containing last portion of NS4, the 26S promoter,  
 the complete BeAn8 structural genes and a portion of  
 the 3' noncoding region.

30 1 CCCAGTAACT CTCTACGGCT GACCTGAATG GACTGTAACG  
 TAGTTCAGTC

35 51 CGCAACCATG TTCCCTTACC AATCACCAAT GTTTCCAATG  
 CAACCAGCGC

40 101 CTTTCGCAA CCCGTACGCT CCTCCTAGAA GACCGTGGTT  
 CCCTAGAAC

45 151 GATCCCTTCT TAGCCATGCA GGTGCAGGAG TTGGCCCGAT  
 CAATGGCGAG

50 201 CTTGACGTTT AACACAGCGTC GAGATACGCC ACCCGAGGGG  
 CCACCTGCTA

251 AGAAGAAGCG TAAGGAGCCT CAACAGCAGG TAGCTCAGGC  
GCAGGGTTAAG

5 301 AAAAAGAACG GAAAACCGAA GAAGAAGAAA AGTAACGGAG  
CACCACCCCC

10 351 AAAAAATCAG AAGAGCACCA AGAAGAAGAC CAATAAGAAA  
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15 401 GACAACGGAT GGTTATGAAG TTAGAATCAG ACAAAACATT  
TCCTATTTG

20 451 CTGGATGGAA AAATTAATGG ATATGCTTGC GTTGTGGAG  
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25 501 TCGACCCATG CACGTGGAAG GCAAAATTGA CAATGAAACT  
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30 551 TGAAGACGAA GAAGGCATCC AAATACGACC TAGAGTATGC  
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35 601 CAAAGCATGC GAGCAGATAAC CTTAAATAC ACACATGATA  
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40 651 GTATTATAAT TGGCATCACG GCGCCGTGCA GTATGAAAAT  
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45 701 CGGTGCCGAA AGGTGTGGGA GCGAAAGGAG ACAGTGGACG  
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65 901 TGTCTTCTCG CCGATGTTAC GTTCCCTTGT TCCACTCCAC  
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1101 GTACATGGCC AAGTGTGTGC GGTGTGCCGT TGGAAGTTGC  
CACAGCCGA

5 1151 TCGCTATAGA AGCAGTAAGA AGCGACGGGC ATGACGGCTA  
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10 1251 AGTTATGAGG TATAATCTGT ATGGCAAGAT CGTAGAAGTT  
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15 1301 AGGTTTCATT ACACACATCT CGGCCTTGCC ACATTATTGA  
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3601 TGTACGAAGG TACTCACATG TAAAGGCGAC TGTACATCCGC  
50 CTAAAGACCA

3651 TATTGTGACG CACCCCCAGC ACCACGCCA GACATTACA  
GCTGCGGTAT

3701 CCAAGACCGC TTGGACGTGG TTAACGTCAC TCTTAGGAGG  
5 GTCAGCAGTA

3751 ATTATTATAAA TTGGCCTTGT ATTAGCAACT GTTGTGCTA  
TGTATGTGCT

10 3801 GACCAACCAG AAACATAATT AGACCACAGC AGCGATTGGA  
AAGCTGCCTA

3851 TTAGAACAT GTAGCGGCAA TTGGCAAGCC GCCTATAAAT  
15 GTTTAGCAGC

3901 AATTGGCAAG CTGCATATAT AAATTACCTA GC GGCAATTG  
GCACGCCGCT

3951 TATAAAATT TTATTTCTT TTACCAATAA TTGGATTGG  
20 TTTTTAATAT

4001 TTC

25

30

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40

What is claimed is:

1. A DNA comprising an isolated and purified western equine encephalitis (WEE) virus cDNA fragment  
5 coding for infectious western equine Encephalitis virus genome.
- 10 2. A DNA according to claim 1 wherein said WEE cDNA fragment is operably linked to a promoter such that said cDNA is transcribed.
- 15 3. The DNA according to claim 1, wherein said cDNA fragment contains a deletion in the E3-E2 cleavage site.
- 20 4. The DNA according to claim 3, wherein said cDNA fragment deletion is 5 amino acids at the E2-E2 cleavage site said amino acids being Arg Arg Pro Lys Arg.
- 25 5. The DNA according to claim 4 wherein said cDNA fragment further contains a suppressor mutation.
- 30 6. The DNA according to claim 5 wherein said suppressor mutation is a substitution of glutamic acid at codon 182 of E2 to lysine.
7. The DNA according to claim 5 wherein said suppressor mutation is a substitution of glutamic acid at codon 181 of E2 to lysine.
8. The DNA according to claim 2 wherein said promoter is T7 promoter of pBluescript KS and said DNA is pWE2000.

9. An infectious WEE virus RNA transcript encoded by the cDNA fragment of claim 1.

5 10. A DNA according to claim 5 wherein said WEE cDNA fragment is operably linked to a promoter such that said cDNA is transcribed.

10 11. An attenuated WEE virus RNA transcript encoded by the cDNA fragment of claim 5.

12. A DNA according to claim 6 wherein said WEE cDNA fragment is operably linked to a promoter such that said cDNA is transcribed.

15 13. An attenuated WEE virus RNA transcript encoded by the cDNA fragment of claim 6.

20 14. A DNA according to claim 7 wherein said WEE cDNA fragment is operably linked to a promoter such that said cDNA is transcribed.

15. An attenuated WEE virus RNA transcript encoded by the cDNA fragment of claim 7.

25 16. Infectious WEE virus particles containing an RNA transcript according to claim 9.

30 17. Attenuated WEE virus particles containing an RNA transcript according to claim 11.

18. Attenuated WEE virus particles containing an RNA transcript according to claim 13.

19. Attenuated WEE virus particles containing an RNA transcript according to claim 15.

20. A live attenuated western equine encephalitis (WEE) virus vaccine comprising attenuated western equine encephalitis virus according to claim 17.

21. A live attenuated western equine encephalitis (WEE) virus vaccine comprising attenuated Western Equine Encephalitis virus according to claim 18.

22. A live attenuated western equine encephalitis (WEE) virus vaccine comprising attenuated western equine encephalitis virus according to claim 19.

23. A DNA comprising an isolated and purified venezuelan equine encephalitis virus IE variant (VEE IE) cDNA fragment coding for infectious venezuelan equine encephalitis virus IE variant virus genome.

24. A DNA according to claim 23, wherein said cDNA fragment has the sequence of SEQ ID NO:2 or a portion thereof, or an allelic portion thereof.

25. The DNA according to claim 23, wherein said cDNA fragment contains a deletion in the E3-E2 cleavage site.

26. A DNA according to claim 25, wherein said cDNA fragment contains a deletion according to

claim 25 wherein said deletion is four amino acids said amino acids being Arg Gly Lys Arg.

27. A DNA fragment according to claim 26  
5 wherein said VEE IE cDNA further contains a suppressor mutation.

28. A DNA according to claim 23 wherein said VEE IE cDNA fragment is operably linked to a promoter  
10 such that said cDNA is transcribed.

29. An infectious VEE IE virus RNA transcript encoded by the cDNA fragment of claim 23.

15 30. A DNA according to claim 27 wherein said VEE IE cDNA fragment is operably linked to a promoter such that said cDNA is transcribed.

20 31. An attenuated VEE IE virus RNA transcript encoded by the cDNA fragment of claim 27.

32. Infectious VEE IE virus particles containing an RNA transcript according to claim 29.

25 33. Attenuated VEE IE virus particles containing an RNA transcript according to claim 31.

30 34. A live attenuated venezuelan equine encephalitis virus IE variant (VEE IE) virus vaccine comprising attenuated venezuelan equine encephalitis virus IE variant virus according to claim 33.

35 35. A bivalent alphavirus vaccine comprising live attenuated western equine encephalitis (WEE) virus comprising an attenuating mutation and live

attenuated venezuelan equine encephalitis virus IE variant (VEE IE) comprising an attenuating mutation.

36. A bivalent alphavirus vaccine according  
5 to claim 35 wherein said live attenuated western  
equine encephalitis virus is chosen from the group  
consisting of attenuated WEE comprising a deletion in  
the E3-E2 cleavage site and a substitution of glutamic  
acid at codon 182 of E2 to lysine, and attenuated WEE  
10 comprising a deletion in the E3-E2 cleavage site and a  
substitution of glutamic acid at codon 181 of E2 to  
lysine.

37. An attenuated western equine  
15 encephalitis virus wherein said virus comprises an  
attenuating mutation selected from the group  
consisting of: a C to T change at nucleotide 7 of the  
5' noncoding region of the WEE genome, a A to G change  
at nucleotide 13 of the 5' noncoding region of the WEE  
20 genome, a T to A change at nucleotide 25 of the 5'  
noncoding region of the WEE genome, and a deletion of  
an A at nucleotide 22 of the 5' noncoding region of  
the WEE genome.

25 38. A pharmaceutical formulation comprising  
attenuated WEE virus particles according to claim 17  
in an effective immunogenic amount in a  
pharmaceutically acceptable carrier.

30 39. A pharmaceutical formulation comprising  
attenuated WEE virus particles according to claim 18  
in an effective immunogenic amount in a  
pharmaceutically acceptable carrier.

40. A pharmaceutical formulation comprising \* attenuated WEE virus particles according to claim 19 in an effective immunogenic amount in a pharmaceutically acceptable carrier.

5

41. A pharmaceutical formulation comprising attenuated VEE IE virus particles according to claim 33 in an effective immunogenic amount in a pharmaceutically acceptable carrier

10

42. An attenuated chimeric virus comprising nonstructural sequences from a first alphavirus and structural sequences from a second alphavirus resulting in attenuation of the second alphavirus.

15

43. An attenuated chimeric virus according to claim 42 wherein wherein said first alphavirus is western equine encephalitis virus and said second alphavirus is chosen from the group consisting of:

20 Sindbis virus, Aura virus, Barmah Forest virus, Bebaru Cabassou virus, Chikungunya virus, Everglades virus, Fort Morgan virus, Getah virus, Highlands J virus, Kyzylagach virus, Mayaro virus, Middelburg virus, Mucambo virus, Ndumu virus, O'nyong-nyong virus,  
25 Pixuna virus, Ross River virus, Sagiyma virus, Semliki Forest virus, SAAR87 virus, Tonate virus, Una virus, venezuelan equine encephalitis virus, and Whataroa virus.

30

44. An attenuated second virus according to claim 43 wherein said second alphavirus is eastern equine encephalitis virus.

35 45. An attenuated chimeric second virus according to claim 42 wherein said first alphavirus is

Venezuelan equine encephalitis virus variant IE and  
said second alphavirus is chosen from the group  
consisting of: venezuelan equine encephalitis virus,  
western eqine encephalitis virus, eastern equine  
5 encephalitis virus, Sindbis virus, Aura virus, Barmah  
Forest virus, Bebaru Cabassou virus, Chikungunya  
virus, Everglades virus, Fort Morgan virus, Getah  
virus, Highlands J virus, Kyzylagach virus, Mayaro  
virus, Middelburg virus, Mucambo virus, Ndumu virus,  
10 O'nyong-nyong virus, Pixuna virus, Ross River virus,  
Sagiyama virus, Semliki Forest virus, SAAR87 virus,  
Tonate virus, Una virus and Whataroa virus.

46. An attenuated chimeric second virus  
15 according to claim 44 wherein said second virus is  
venezuelan equine encephalitis virus variant IA.

47. An attenuated chimeric second virus  
according to claim 42 wherein said first alphavirus is  
20 venezuelan equine encephalitis virus variant IA and  
said second alphavirus is chosen from the group  
consisting of: Venezuelan equine encephalitis virus,  
western eqine encephalitis virus, eastern equine  
encephalitis virus, Sindbis virus, Aura virus, Barmah  
25 Forest virus, Bebaru Cabassou virus, Chikungunya  
virus, Everglades virus, Fort Morgan virus, Getah  
virus, Highlands J virus, Kyzylagach virus, Mayaro  
virus, Middelburg virus, Mucambo virus, Ndumu virus,  
O'nyong-nyong virus, Pixuna virus, Ross River virus,  
30 Sagiyama virus, Semliki Forest virus, SAAR87 virus,  
Tonate virus, Una virus, and Whataroa virus.

48. An attenuated chimeric second virus  
according to claim 47 wherein said second virus is  
35 venezuelan equine encephalitis virus variant IE.

49. An attenuated chimeric second virus according to claim 47 wherein said second virus is Venezuelan Equine Encephalitis virus variant IIIA.

5

50. An attenuated virus vaccine comprising chimeric virus according to claim 42 wherein said vaccine is directed against said second alphavirus.

10

51. An inactivated western equine encephalitis virus (WEE) vaccine comprising attenuated WEE according to claim 17 wherein said attenuated WEE is inactivated.

15

52. An inactivated western equine encephalitis virus (WEE) vaccine comprising attenuated WEE according to claim 18 wherein said attenuated WEE is inactivated.

20

53. An inactivated western equine encephalitis virus (WEE) vaccine comprising attenuated WEE according to claim 19 wherein said attenuated WEE is further inactivated.

25

54. An inactivated venezuelan equine encephalitis virus variant IE (VEE IE) vaccine comprising attenuated VEE IE according to claim 33 wherein said attenuated VEE IE is further inactivated.

30

55. An inactivated eastern equine encephalitis virus (EEE) vaccine comprising attenuated EEE according to claim 44 wherein said attenuated virus is further inactivated.

56. An inactivated venezuelan equine encephalitis virus variant IA (VEE/IA) vaccine comprising attenuated VEE/IA according to claim 46 wherein said attenuated virus is further inactivated.

5

57. An inactivated venezuelan equine encephalitis virus variant IIIA (VEE/IA) vaccine comprising attenuated VEE IIIA according to claim 49 wherein said attenuated virus is further inactivated.

10

58. An inactivated venezuelan equine encephalitis virus variant IE (VEE IE) vaccine comprising attenuated VEE IE according to claim 48 wherein said attenuated virus is further inactivated.

15

59. A method for expressing a protein said method comprising cloning a gene encoding said protein into a an attenuated virus replicon, said replicon chosen from the group consisting essentially of attenuated WEE and attenuated VEE IE wherein transcription of said replicon yields RNA capable of infecting a cell in which said protein is to be expressed.

25

60. A method for the diagnosis of western equine encephalitis virus (WEE) infection comprising the steps of :

30

(i) contacting a sample from an individual suspected of having a WEE infection with all or a unique portion of WEE; and

(ii) detecting the presence or absence of a WEE infection by detecting the presence or absence of a complex formed between WEE and antibodies specific therefor in the sample.

61. A method for the diagnosis of venezuelan equine encephalitis virus variant IE (VEE IE) infection comprising the steps of :

5 (i) contacting a sample from an individual suspected of having a VEE IE infection with all or a unique portion of VEE IE; and

10 (ii) detecting the presence or absence of a VEE IE infection by detecting the presence or absence of a complex formed between VEE IE and antibodies specific therefor in the sample.

62. A method for the diagnosis of western equine encephalitis virus (WEE) from a sample using 15 the polymerase chain reaction, said method comprising:

(i) extracting RNA from the sample;  
(ii) reverse transcribing the RNA of (i) to DNA;  
(iii) contacting said DNA with  
20 (a) at least four nucleotide triphosphates,  
(b) a primer that hybridizes to WEE DNA, and  
(c) an enzyme with polynucleotide synthetic activity,

under conditions suitable for the hybridization and extension of said first primer by said enzyme, 25 whereby a first DNA product is synthesized with said DNA as a template therefor, such that a duplex molecule is formed;

30 (iv) denaturing said duplex to release said first DNA product from said DNA;  
(v) contacting said first DNA product with a reaction mixture comprising:

(a) at least four nucleotide triphosphates,  
(b) a second primer that hybridizes to said first DNA, and

(c) an enzyme with polynucleotide synthetic \* activity,

under conditions suitable for the hybridization and extension of said second primer by said enzyme,

5 whereby a second DNA product is synthesized with said first DNA as a template therefor, such that a duplex molecule is formed;

(vi) denaturing said second DNA product from said first DNA product;

10 (vii) repeating steps iii-vi for a sufficient number of times to achieve linear production of said first and second DNA products;

(viii) fractionating said first and second DNA products generated from said WEE DNA; and

15 (ix) detecting said fractionated products for the presence or absence of WEE in a sample.

63. A method for the diagnosis of venezuelan equine encephalitis variant IE virus (VEE IE) from a sample using the polymerase chain reaction, said 20 method comprising:

(i) extracting RNA from the sample;

(ii) reverse transcribing said RNA of (i) to DNA;

(iii) contacting said DNA with

25 (a) at least four nucleotide triphosphates,

(b) a primer that hybridizes to VEE IE DNA,

and

(c) an enzyme with polynucleotide synthetic activity,

30 under conditions suitable for the hybridization and extension of said first primer by said enzyme, whereby a first DNA product is synthesized with said DNA as a template therefor, such that a duplex molecule is formed;

(iv) denaturing said duplex to release said first DNA product from said DNA;

(v) contacting said first DNA product with a reaction mixture comprising:

5 (a) at least four nucleotide triphosphates,

(b) a second primer that hybridizes to said first DNA, and

(c) an enzyme with polynucleotide synthetic activity,

10 under conditions suitable for the hybridization and extension of said second primer by said enzyme, whereby a second DNA product is synthesized with said first DNA as a template therefor, such that a duplex molecule is formed;

15 (vi) denaturing said second DNA product from said first DNA product;

(vii) repeating steps iii-vi for a sufficient number of times to achieve linear production of said first and second DNA products;

20 (viii) fractionating said first and second DNA products generated from said VEE IE DNA; and

(ix) detecting said fractionated products for the presence or absence of VEE IE in a sample.

25 64. A method for providing protective immunity against a second alphavirus to individuals with pre-existing immunity to a first alphavirus said method comprising administering to said individuals an effective amount of live attenuated second alphavirus.

30

65. A method for providing protective immunity against a second alphavirus according to claim 64, wherein said live attenuated second alphavirus is attenuated western equine encephalitis

35 virus.

66. The method of claim 65 wherein said western equine encephalitis (WEE) virus is chosen from the group consisting of: attenuated WEE having a 5 substitution of glutamic acid at codon 181 of E2 to lysine, attenuated WEE having a substitution of glutamic acid at codon 181 of E2 to lysine, attenuated WEE having a C to T change at nucleotide 7 of the 5' noncoding region of the WEE genome, 10 attenuated WEE having a A to G change at nucleotide 13 of the 5' noncoding region of the WEE genome, attenuated WEE having a T to A change at nucleotide 25 of the 5' noncoding region of the WEE genome, and attenuated WEE having a deletion of an A at nucleotide 15 22 of the 5' noncoding region of the WEE genome.

67. A method for providing protective immunity against a second alphavirus according to claim 64, wherein said live attenuated second 20 alphavirus is attenuated venezuelan equine encephalitis virus variant IE.

68. A WEE infection diagnostic kit comprising primers specific for WEE and ancillary 25 reagents suitable for use in detecting the presence or absence of WEE in a mammalian sample.

69. A VEE IE infection diagnostic kit comprising primers specific for VEE IE and ancillary 30 reagents suitable for use in detecting the presence or absence of VEE IE in a mammalian sample.

70. An isolated and purified Venezuelan equine encephalitis variant IIIA cDNA encoding the VEE 35 IIIA structural genes.

71. An amino acid fragment encoded by the  
cDNA fragment according to claim 70.

5 72. A polypeptide encoded by the amino acid  
fragment according to claim 71.

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## Assembly : module WE3'-17 1 / 7

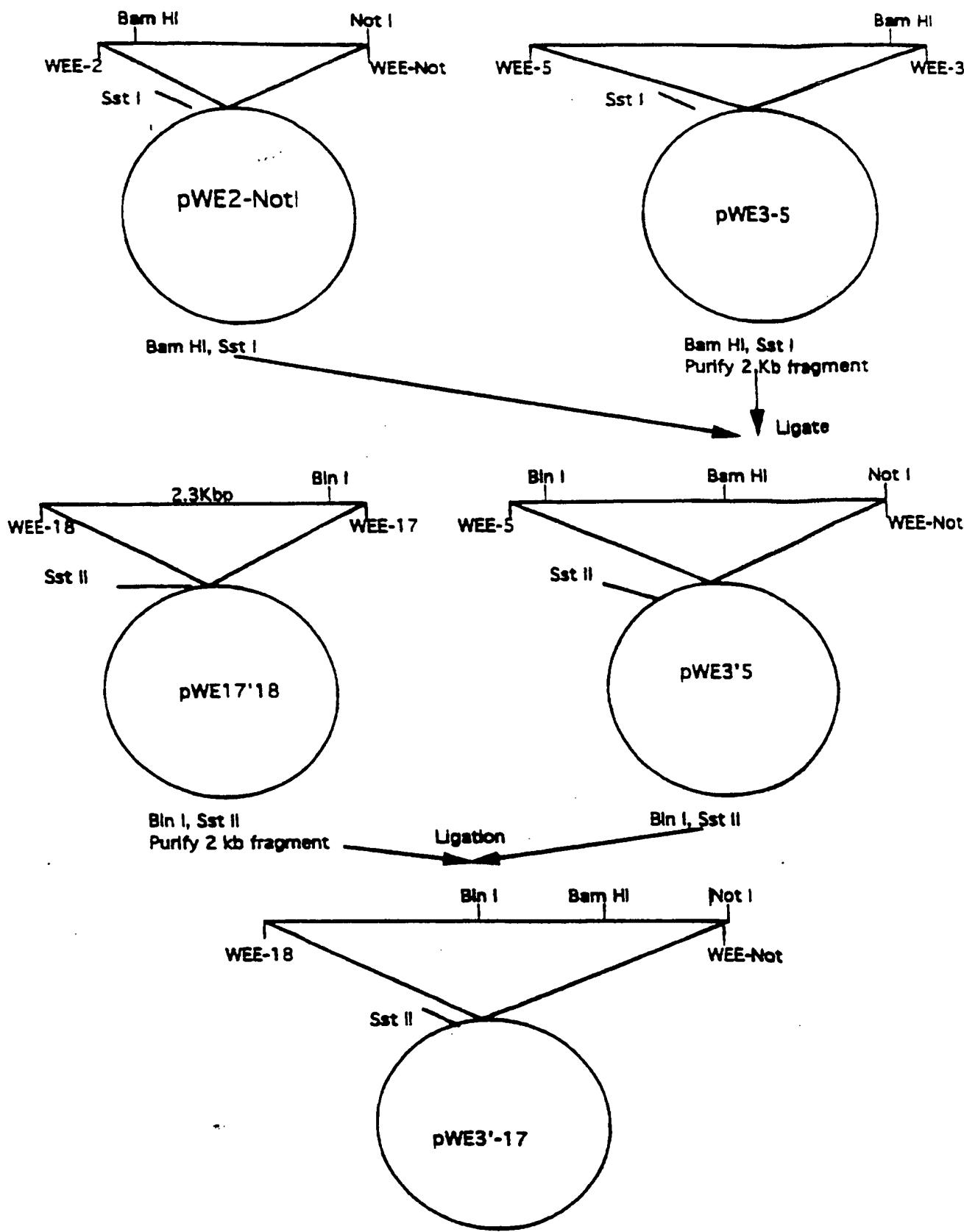


FIGURE 1A

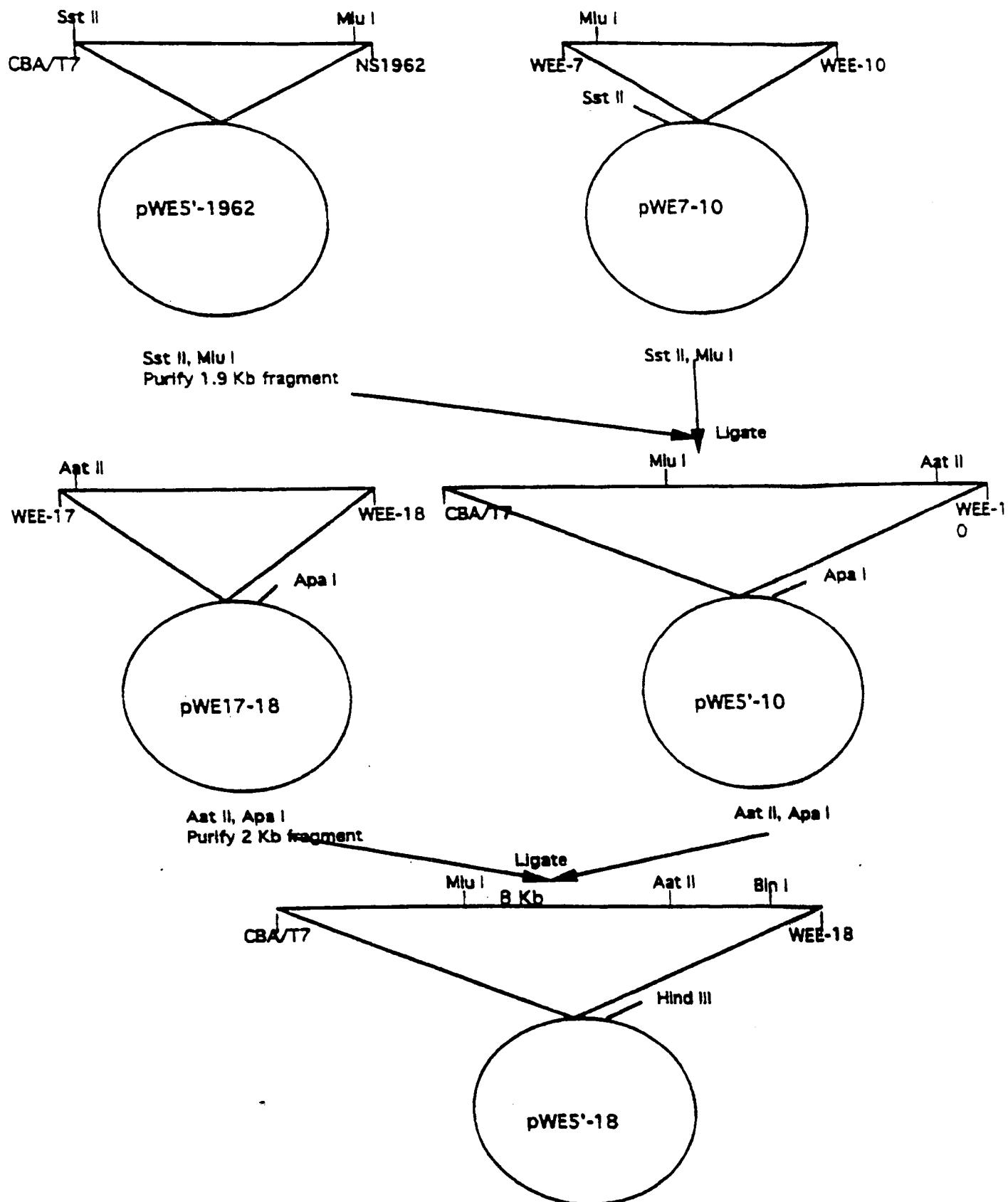
Assembly c noc  $\rightarrow$  pWE5'-18 2 / 7

FIGURE 1B

3 / 7

Assembly of pWE2000 full length cDNA clone of western equine encephalitis virus from modules pWES'-18 and pWE3'-17

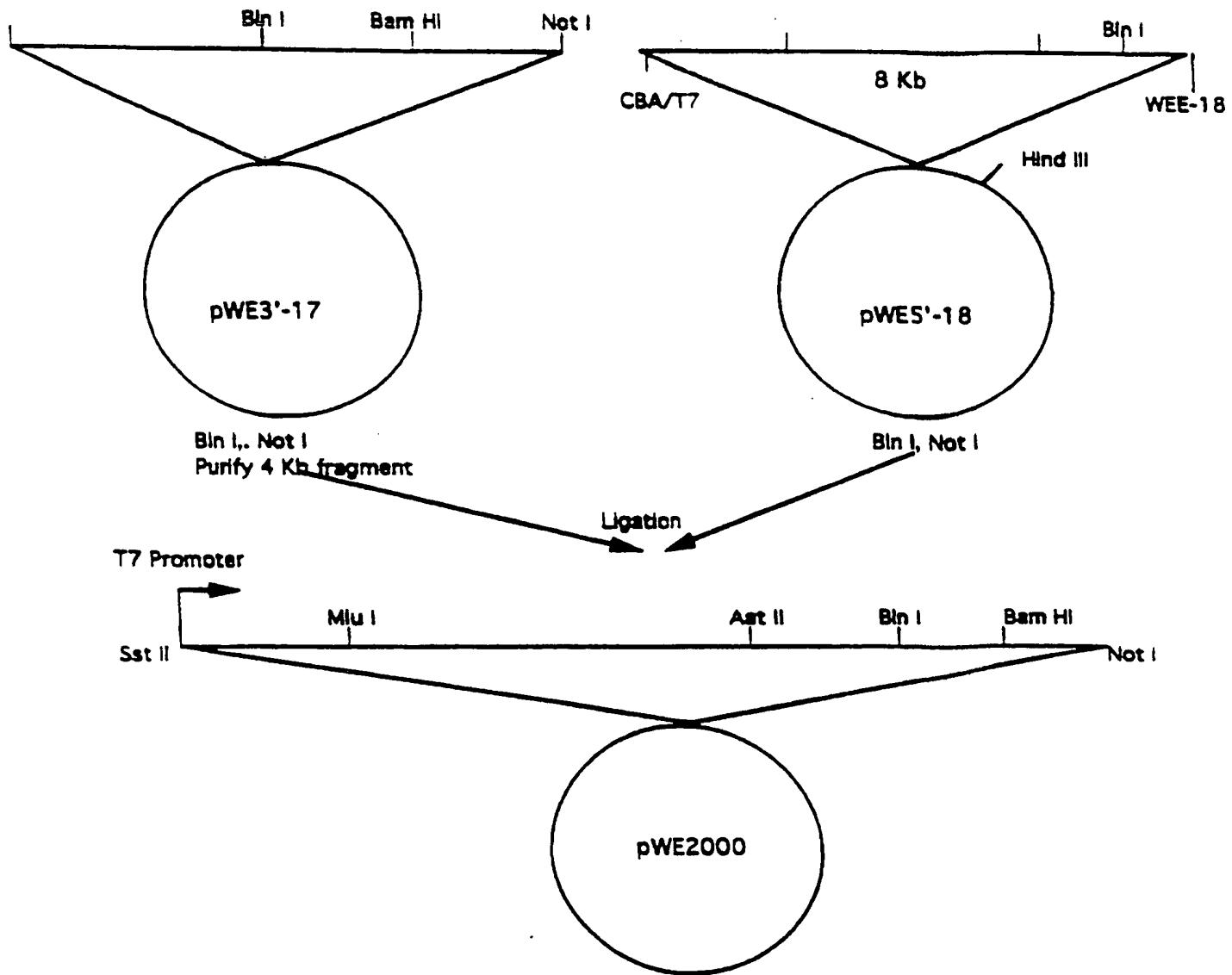


FIGURE 1C

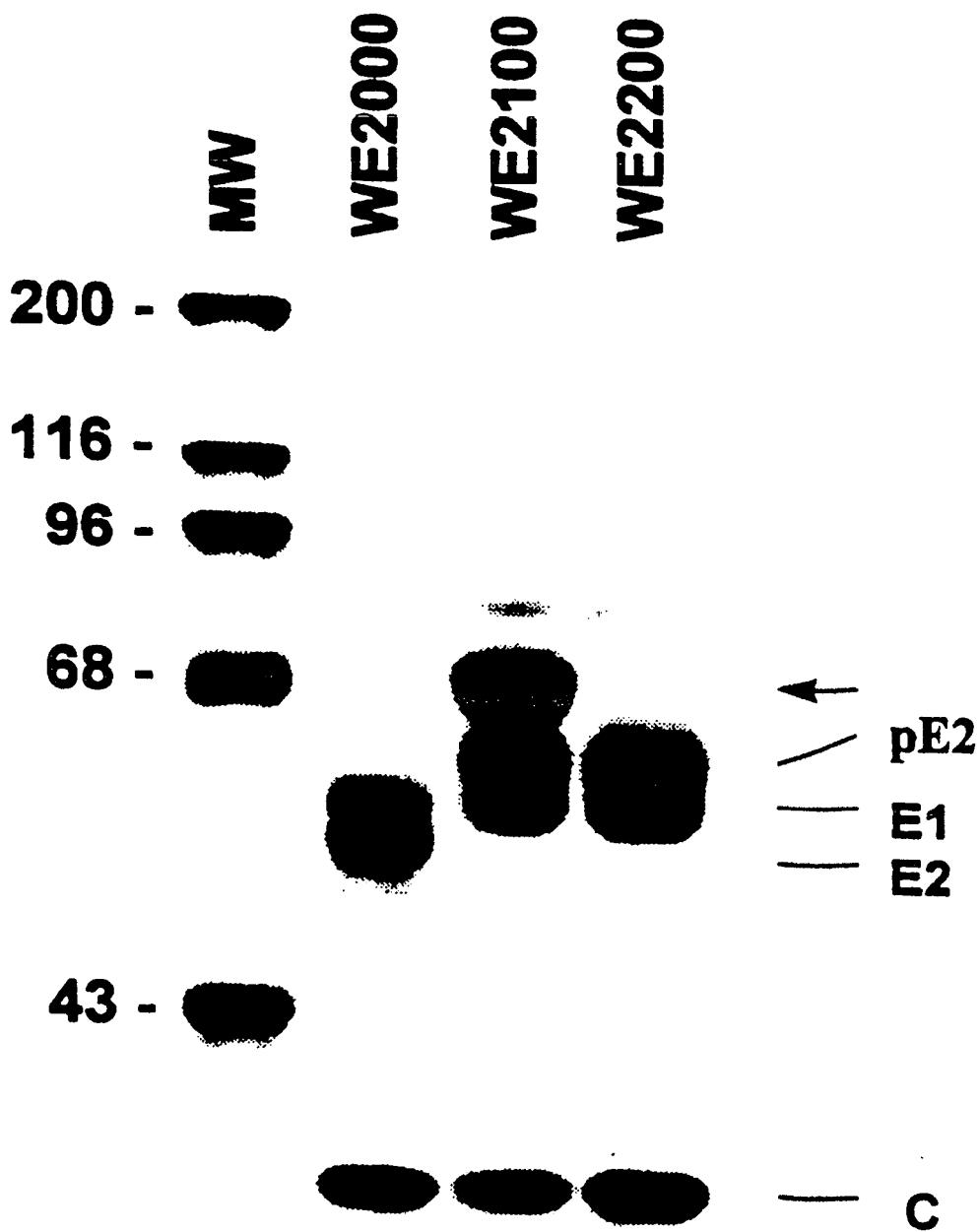


FIGURE 2

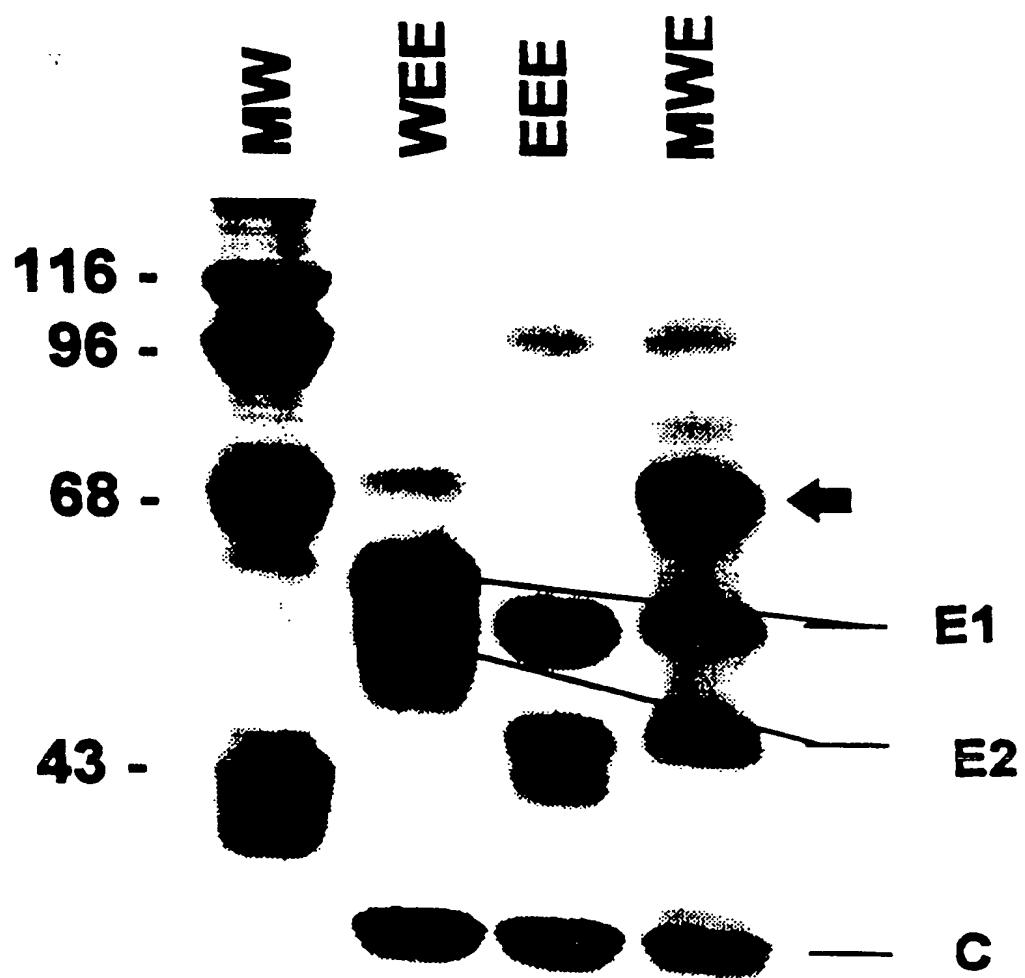
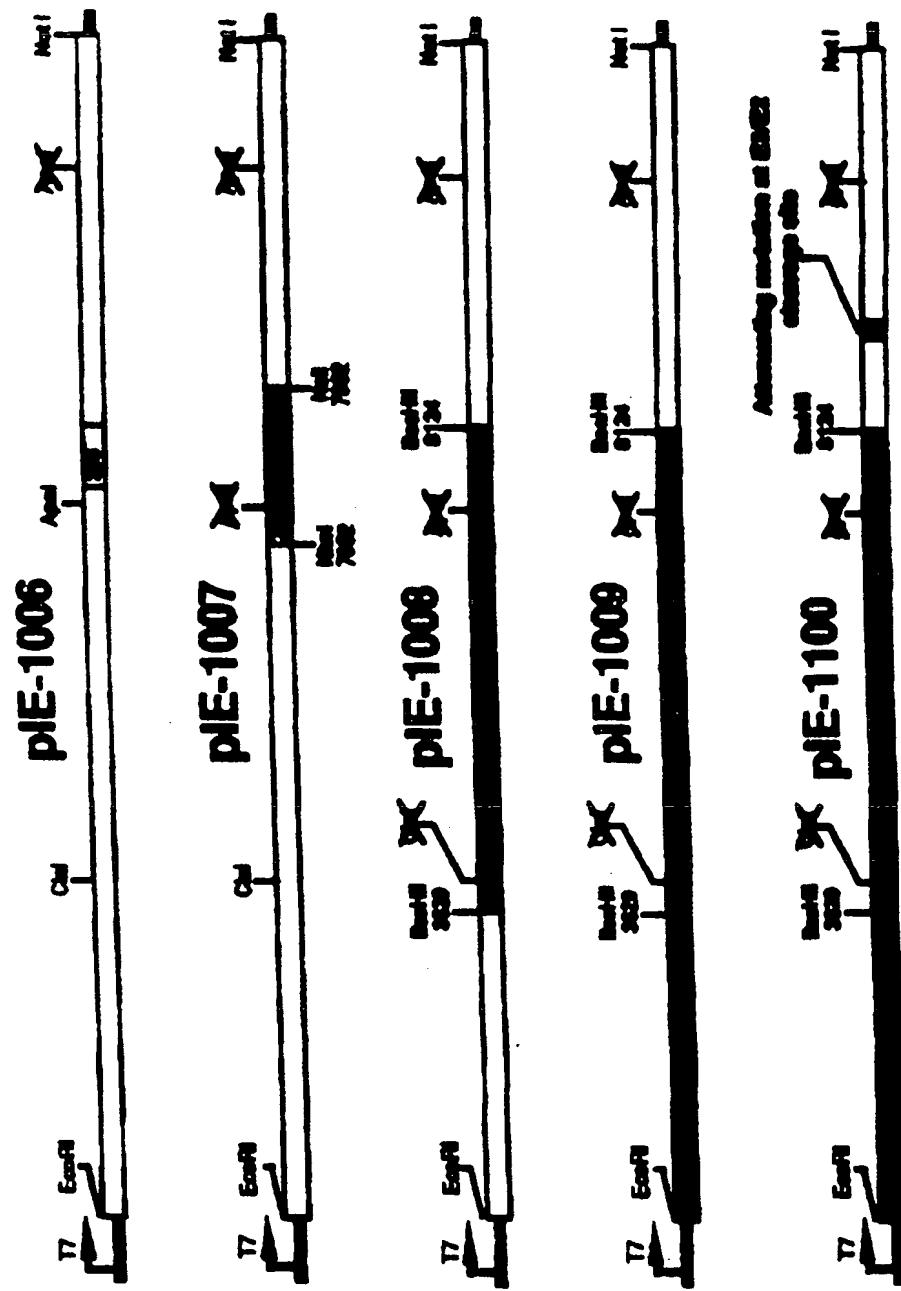


FIGURE 3

**Figure 4**



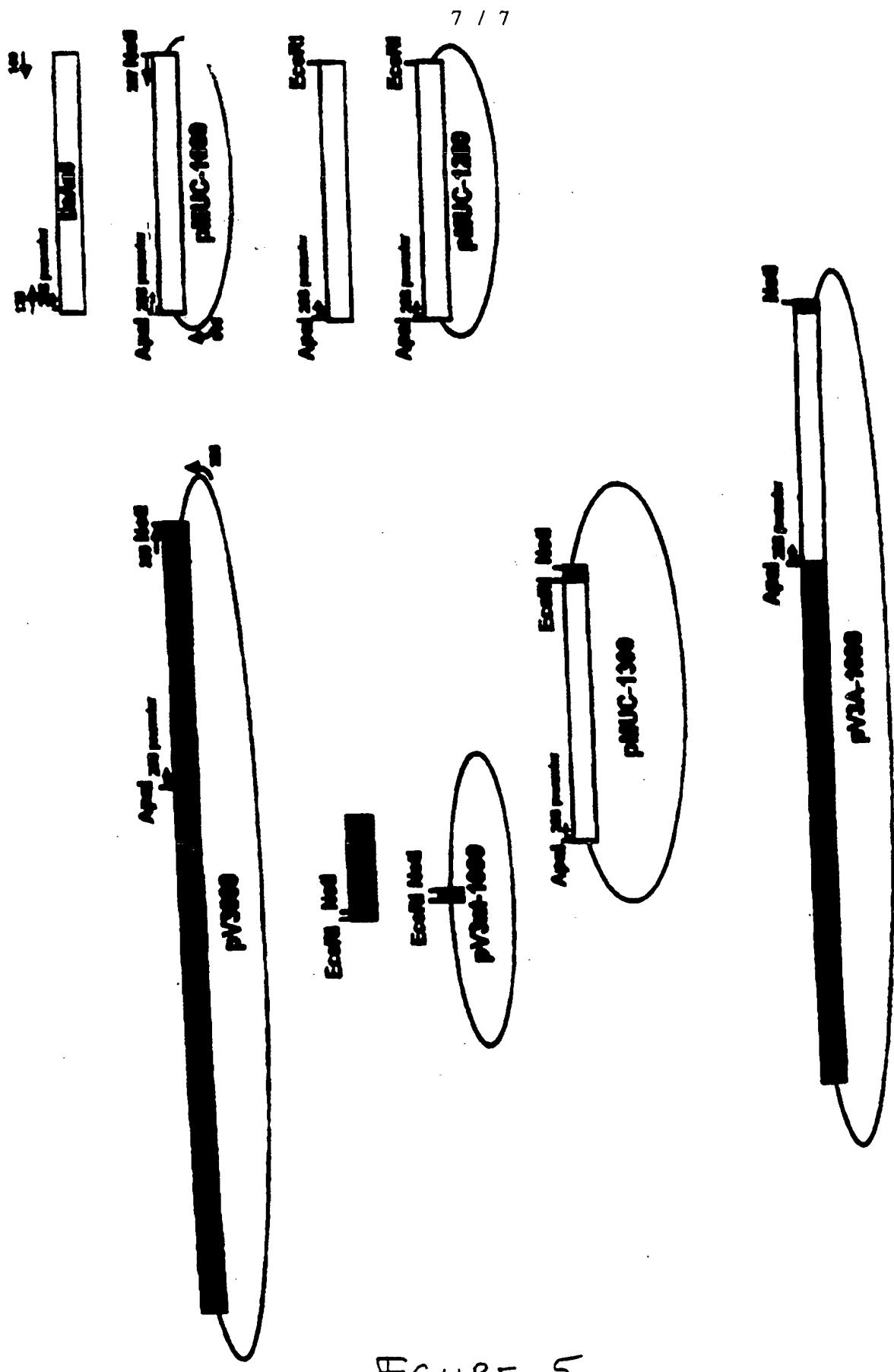


FIGURE 5

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/10645

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/40, 7/01, 7/04, 15/86; A61K 39/193.

US CL :536/23.72; 435/235.1, 236, 69.1, 172.3; 424/205.1, 218.1

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.72; 435/235.1, 236, 69.1, 172.3; 424/205.1, 218.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Aps, Biosis, Cab, Derwent WPI. Search terms: western, equine(w)enceph?, attenuat?, venezuelan, genom?, ma. clon?, sequenc?, dna, dnas, "E2", "E3", wee.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FRAIZER, G. et al. Isolation and preliminary characterization of mutants of Western equine encephalomyelitis virus with altered virulence in chickens. Biological Abstracts. 1985. Vol. 80, No. 5, page AB-506, Abstract no. 41179, see entire abstract.	16
Y	US 5,185,440 A (DAVIS et al) 09 February 1993, see entire document, particularly column 7, line 66 through column 8, line 1.	1-3, 8, 9, 16, 59
Y	US 5,505,947 A (JOHNSTON et al) 09 April 1996, see particularly column 3, lines 50-54, Abstract, and Examples 1 and 2.	3, 59

 Further documents are listed in the continuation of Box C.

See patent family annex.

Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

27 AUGUST 1998

Date of mailing of the international search report

06 OCT 1998

Name and mailing address of the ISA/US  
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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/10645

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WEAVER, S.C. et al. A comparison of the Nucleotide Sequences of Eastern and Western Equine Encephalomyelitis Viruses with Those of Other Alphaviruses and Related RNA Viruses. <i>Virology</i> . 1993. Vol. 197, pages 375-390. See figures 3 and 4.	1-3, 8, 9, 16, 59
Y	HAHN, C.S. et al. Western equine encephalitis virus is a recombinant virus. <i>Proceedings of the National Academy of Sciences USA</i> . August 1988. Vol. 85, p. 5997-6001. See Figure 1.	1-3, 8, 9, 16, 59
A	STRAUSS, J.H. et al. The Alphaviruses: Gene Expression, Replication, and Evolution. <i>Microbiological Reviews</i> . September 1994. Vol. 58, No. 3, pages 491-562.	1-22, 37-40, 51-53, 59

**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/US98/10645

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-22, 37-40, 51-53, 59

**Remark on Protest**

The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/10645

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group 1, claims 1-22, 37-40, 51-53, 59, drawn to a first product DNA encoding an infectious genome of Western Equine Encephalitis (WEE) virus, RNA and virus encoded by DNA, vaccines and pharmaceuticals comprising virus encoded, and methods of use.

Group 2, claims 23-34, 41, 54, 59 drawn to second product DNA encoding an infectious genome of Venezuelan Equine Encephalitis IE variant (VEE IE) virus, RNA and virus encoded by DNA, vaccines and pharmaceuticals comprising virus encoded, and methods of use.

Group 3, claims 35-36, drawn to third product, combination vaccine comprising attenuated WEE and attenuated VEE IE

Group 4, claims 42-50, 55-58, drawn to fourth product, chimeric alphavirus and vaccine.

Group 5, claims 62, 68, drawn to fifth product, WEE specific primer kit, and method of use.

Group 6, claims 63, 69, drawn to sixth product, VEE IE specific primer kit, and method of use

Group 7, claims 70-72, drawn to seventh product, DNA encoding structural genes of VEE variant IIIA and products encoded.

Group 8, claims 60, drawn to method of diagnosing WEE using antigen.

Group 9, claims 61, drawn to method of diagnosing VEE IE using antigen.

Group 10, claims 64-67, drawn to method of vaccinating immune individual using a second alphavirus.

The inventions listed as Groups 1-10 do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

In groups 1 and 2, the special technical feature in each case is the DNA encoding an infectious genome of a particular virus; since groups 1 and 2 pertain to two distinct viruses, they involve different special technical features.

Group 3 does not have the corresponding special technical feature, because the attenuated viruses required in group 3 are not limited to those made by using the DNAs of groups 1 or 2; for example claim 35 encompasses combination vaccines using viruses attenuated by propagation in culture.

Group 4 does not have the corresponding special technical feature, because it is not limited to either of the viruses encoded by the DNAs of group 1 or group 2; for example claim 42 encompasses a chimera made from Sindbis and Semliki Forest viruses.

Groups 5 and 6 do not have the corresponding special technical feature, because they require small, specific primers, not infectious full-length genomic materials.

Group 7 does not have the corresponding special technical feature, because it is drawn to a sub-genomic fragment of a third virus.

Groups 8 and 9 do not have the corresponding special technical feature, because they are not limited to materials of groups 1 or 2; for example claim 60 encompasses a method using viral antigens produced by propagation of native WEE in standard tissue culture.

Group 10 does not have the corresponding special technical feature because it is not limited to the viruses made using the DNAs of groups 1 or 2; for example claim 64 encompasses a method of vaccinating a Sindbis-immune monkey

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US98/10645

against Semliki Forest virus.

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